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ALBERT J. KLUYVER

PROFESSOR OF MICROBIOLOGY
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EDITORS:
MARIE P. LÖHNIS
WAGENINGEN

L. H. C. PERQUIN
BREDA

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Waarde Kluyver,

Het feit dat de Ned. Vereeniging voor Microbiologie dezen datum, de 25ste verjaardag van je aanvaarding van de leerstoel in de Microbiologie aan de Technische Hoogeschool in Delft, heeft uitgekozen om zichzelf te eeren door dit nummer van haar tijdschrift Antonie van Leeuwenhoek aan je op te dragen, is slechts een pretext!

Inderdaad, niets onderscheidt het getal 25 van andere getallen en als we de slechte smaak adden gehad je te raadplegen aangaande de wenschelijkheid van deze huldiging, dan zou je zonder twijfel getracht hebben je veto uit te spreken over ons voornemen op deze en vele andere gronden.

En dus hebben we je niet geraadpleegd. Maar we zijn graag bereid, het getal 25 uit onze overwegingen te bannen als je ons wilt toestaan onze zin te doen, alleen omdat het ons een genoegen is deze gelegenheid (elke gelegenheid!) aan te grijpen om je te danken uit naam van bacteriologen van elke pluimage, in Nederland en daar buiten, voor wat je gedaan hebt ter bevordering van onze wetenschap, voor de bacteriologische opvoeding van zoo vele jonge studenten en voor de gastvrijheid en blijvende vriendschap, waarmee je tallooze collega's uit alle landen hebt verrijkt.

Een uitstekend geleerde eeren is een prettige taak, zij het een landgenoot of een vreemdeling, en je zult daarom niet verbaasd zijn te vernemen, dat onze oproep tot een groot aantal collegae in Nederland en elders overal hartelijke weerklank vond en dat het gemakkelijk genoeg zou zijn geweest een bundel uit te geven, dubbel zoo groot als deze, indien de betreurenswaardige papierschaarschte geen hinderpaal was geweest.

Sta ons dus toe je te zeggen, dat de wijze waarop je gedurende 25 jaar Beijerinck's erfenis beheerd hebt (zonder twijfel geen gemakkelijke taak!) de grootste waardeering gevonden heeft in de geheele wetenschappelijke wereld en neem het ons niet kwalijk als wij, van de Microbiologische Vereeniging, eenmaal in de 25 jaar onszelf het genoegen doen je dit te zeggen, door het verzamelen van een reeks wetenschappelijke artikelen van je vele vrienden en door je deze bundel aan te bieden als een bewijs van onze bewondering, onze dankbaarheid en onze vriendschap.

Jan Smit,

Voorzitter.

1922 — JANUARY, 18th — 1947

Dear K l u y v e r,

The fact that the Netherlands Society of Microbiology has chosen this date, the 25th anniversary of your taking the Chair of Microbiology at the Technical University at Delft, to honour itself in dedicating this volume of her periodical Antonie van Leeuwenhoek to you, is but a pretext!

In fact, nothing in particular distinguishes the figure 25 from others, and if we should have had the bad taste of consulting you on the desirability of this commemoration, you would undoubtedly have tried to veto our intention on this and many other grounds.

So we did not consult you. But we are quite willing to discard the number 25 from our consideration if you will allow us to have our way, only because it is our pleasure to seize this opportunity (any opportunity!) to thank you in the name of bacteriologists of every creed, in the Netherlands and abroad, for what you have done to the advancement of our science, for the bacteriological education of so many young students and for the hospitality and lasting friendship, which you have bestowed on numbers of colleagues from all countries.

Honouring an eminent scientist is a pleasant task, be it a fellow-countryman or a foreigner, and so you will not be surprised to hear that our appeal to a great number of colleagues in Holland and abroad was heartily welcomed everywhere, and that it would have been easy enough to issue a volume twice the size of the present one, had not the regrettable paper shortage been prohibitive.

So allow us to say that the manner in which, during 25 years, you have taken charge of B e ij e r i n c k's heritage (by no means an easy task!) has found the warmest appreciation throughout the scientific world, and don't blame us if, once in 25 years, we of the Microbiological Society, take the pleasure of telling you so, by collecting a series of scientific papers from your many friends and offering this volume to you as a token of our admiration, our gratitude and our friendship.

J a n S m i t,

President.

(Institut Pasteur à Brie Comte-Robert).

PRINCIPES DE LA MICROBIOLOGIE OECOLOGIQUE

par

S. WINOGRADSKY

(Reçu le 23 août 1946).

Aperçu historique. L'origine lointaine de cette branche de la grande science microbiologique est à chercher dans les idées de PASTEUR sur le rôle des „infiniment petits" dans la nature. D'autre part, il est évident que la recherche des agents vivants doit être précédée par la connaissance des phénomènes, qui se déroulent dans le milieu naturel. C'est en ce sens que les agrochimistes français — les BOUSSAINGAULT, SCHLOESING et MUNTZ, DÉHÉRAIN et autres — sont à considérer comme initiateurs des recherches agrobiologiques. Par leur études mêmes sur les problèmes agrochimiques, ils ont posé les problèmes agrobiologiques à résoudre. Particulièrement, la découverte par SCHLOESING et MUNTZ de la nature biologique de la Nitrification, phénomène des plus caractéristiques du sol, le plus anciennement connu, le mieux étudié, (BOUSSAINGAULT) a servi de point de départ aux recherches dans la voie pastorienne. La découverte des agents microbiens de la Nitrification en a été le premier pas décisif (1890).

Il est vrai que la découverte des radicicoles des Légumineuses par BEIJERINCK et de la fixation symbiotique a précédé d'une paire d'années celle des Nitrificateurs (1888); mais ce problème d'un genre spécial, sujet d'études des botanistes et des agronomes de langue allemande depuis la moitié du siècle dernier, s'est développé en quelque sorte à part, sans exercer une influence sensible sur le développement des recherches agrobiologiques.

Quant à l'opinion qui voit l'origine de la branche qui nous intéresse dans les travaux de R. KOCH et de son école, époque ou un nombre considérable d'espèces bactériennes a été isolé du sol en culture pure, elle ne me paraît pas soutenable. Comme on le sait, ces études ont été effectuées par des chercheurs qui n'étaient pas au courant des questions agrochimiques, ne s'intéressant qu'à des question d'Hygiène. C'est en partant de ce point de vue que de si nombreuses recherches ont été conduites sur la densité des

germes dans le sol, au moyen du dénombrement de colonies parues sur la gélatine nutritive, méthode qui n'a donné que des résultats décevants en Agrobiologie. Il est vrai, que ces travaux ont apporté des perfectionnements de technique, dont toutes les branches de la Microbiologie ont bénéficié, mais en ce qui concerne spécialement les problèmes touchant à la dynamique des milieux naturels, aucune tentative sérieuse n'a été faite pour les approcher.

Les études sur la fixation anaérobie, avec la découverte d'un premier microbe fixateur d'azote, ont suivi de près celles de la Nitrification (1893), et c'est à cette époque que l'on a commencé à parler de Microbiologie Agricole. „Etait-ce une nouvelle branche douée de sa méthode propre, élaborée spécialement dans le but de s'attaquer à des problèmes agrobiologiques? . . . . On est allé simplement emprunter ses éléments au stock des notions acquises par les études de Microbiologie Générale". (Conférence au premier Congrès de l'Association de la Science du Sol, Rome 1924).

Une période d'une trentaine d'années s'est écoulée, remplie par des recherches sur la Nitrification, l'Ammonification, la Fixation, quelques phénomènes de décomposition, de production d'acide carbonique dans le sol. On isolait des espèces bactériennes, et on étudiait en culture pure leurs caractères morphologiques et physiologiques. Déterminer la densité des germes dans des conditions variées — saisonnières, météorologiques, culturales — restait toujours un sujet favori de recherche. La liste des bacilles isolés en culture pure s'allongeait rapidement, mais sans que l'on puisse se faire une idée assez précise sur leur rôle au sein du milieu naturel. Le sol lui-même, en qualité de milieu peuplé, d'un réservoir d'énergie, ainsi que les modalités de la dégradation de la matière végétale qui y a lieu restaient en dehors du plan de travail.

Tel était dans ses grandes lignes l'état de la Microbiologie, dite Agricole, vers la fin de la seconde décade du siècle courant. Mais depuis ce temps là les recherches sur la Microbiologie des milieux naturels se sont développées rapidement, en prenant un tour plus adéquat au but à atteindre; particulièrement en Amérique, grace aux travaux des Stations Expérimentales Agronomiques. Signalons spécialement ceux de WAKSMAN et de son école, qui ont apporté les notions biochimiques sur le sol arable et autre milieux naturels, sur la composition de leur peuplement microbien et son activité g l o b a l e dans la décomposition de la matière végétale brute, suivie dans toutes ses phases, jusqu'à la formation de l' H u m u s; lequel serait le dernier résidu des activités microbiennes sur cette

matière. Ce sujet pluriséculaire est traité à fond dans l'ouvrage du savant amériquain: Humus, Origin, Chemical composition, Importance in nature, Baltimore, 1936.

Il y a lieu à reconnaître que l'ensemble de ces contributions a nettement tracé les cadres d'une nouvelles discipline microbiologique; et ce qui est important pour son développement, des rapports plus étroits se sont établis entre la Science du sol sous tous ses aspects — physico-chimiques et spécialement pédologiques — et les études agrobiologiques. Cette tendance à la collaboration scientifique a trouvé sa meilleure expression dans la constitution de l'Association Internationale de la Science du Sol, fondée en 1924 au Congrès de Rome.

On comprend que les Microbiologistes intéressés ont cru devoir réagir, en gravitant vers ce nouveau centre d'études collectives et que cette orientation devait diriger leurs études vers les fonctions naturelles de la Microflore authentique; c'est à dire, non modifiée par les conditions qu'elle trouve au laboratoire; autrement dit, sur son Oecologie, étude qui s'impose en premier lieu quand il s'agit de libre existence au sein de la nature.

Or, il est évident que, si le principe de la culture pure est obligatoire pour les études physiologiques et biochimiques, s'il est même la condition *sine qua non* de leur conduite correcte, — il est de nature à rendre impossible toute étude oecologique sérieuse.

Le principe de la culture pure. Discutons-le en peu de mots, en commençant par l'isolation de la souche de son habitat naturel, le sol. Cette opération est effectuée sans ménager les caractères naturels de l'espèce, et non sans recourir, dans des cas difficiles, à des moyens „violents" — tels que température élevée, réaction poussée vers l'acidité ou l'alcalinité, dose de toxique etc.; donc, sans prendre garde de ne pas ébranler la constitution de l'espèce. Ce qui inspire des doutes, si la forme isolée peut être envisagée en toute sûreté, comme identique à la forme d'origine, dont elle provient. Et quant aux cultures de collection, tenues dans nos serres à microbes pendant de longues années, on ne saurait douter qu'elles ne soient plus identifiables à leurs ancêtres sauvages.

„Many of our cultures are worthy of the name of physiological artefacts" remarque si judicieusement le prof. A. J. Kluyver, Proc. Kon. Akad. v. Wet. Amsterdam **35**, 370, 1932.

L'usage d'un micromanupulateur ne pourraît remédier à cet état de chose, du moment que les cultures provenant d'une seule cellule ne présenteraient que la descendance d'un seul Klon, au lieu de la forme moyenne, qui est la forme naturelle. Utile, quand il s'agit de la dissociation de l'espèce, l'usage de cet appareil dans notre cas ne peut conduire qu'à des souches, qui ne sont, à proprement parler, que des artefacts morphologiques, et peut-être encore physiologiques; on ne sauraît en tout cas les identifier avec les souches naturelles.

Quant à la culture pure (quelque soit le milieu, qu'il soit favorable ou non) l'absence de tout germe étranger, donc de toute compétition, y crée nécessairement une ambiance biologique artificielle; et c'est cette même condition qui donne à l'experimentateur libre carrière de forcer la nature de l'espèce, de la plier à des modes d'existence modifiés, en exploitant ses caractères potentiels ou latents, que celle-ci n'aurait eu, peut-être, jamais l'occasion de développer, en dehors de l'ingérence de l'opérateur. Or ce sont les caractères actuels ou réels qui intéressent l'oecologiste, lequel devrait bien se méfier des caractères qui se sont développés en culture pure. Cela d'autant plus, qu'un facteur oecologique des plus important est éliminé par cette dernière; j'entend, la compétition, car c'est elle qui commande la répartition des activités microbiennes dans la nature, et qui règle leur succession au-moyen d'un mécanisme automatique.

On ne saurait nier pourtant, comme on me l'avait déja objecté, que l'on a réussi, en se servant de la méthode classique, à isoler quelques agents importants du sol (nitrification, fixation etc.). Ces succès étaient dûs, comme on le sait, au principe de la culture élective, lequel reste toujours bien utile, tout en n'étant applicable sous sa forme courante qu'à un nombre de cas assez restreint. En somme, les perfectionnements que ce principe avait apporté à la méthode classique ne l'ont pas rendue beaucoup plus apte à servir à des études oecologiques; de sorte que, même dans des cas ou l'on connaissait l'agent actif, elle est restée impuissante à démêler les facteurs de son activité au sein du milieu naturel.

Les critiques que nous venons de formuler feront comprendre les raisons, dont l'auteur s'est inspiré au début même de ses études sur la Microbiologie du sol pour juger la méthode courante inapte à y servir sans modifications assez profondes.

Logiquement, il ne paraissait pas difficile d'imaginer les modifications de principe qu'il serait nécessaire d'y introduire. Ce seraient:

1° renoncer à la culture pure obligatoire,
2 éviter les cultures de collection,
3 emprunter les germes pour les expériences directement au milieu naturel,
4 se garder d'ébranler la constitution de l'espèce par le procédé d'isolement, ainsi que par la culture sur un milieu, auquel elle n'est pas adaptée,
5° Chercher, dès le début des expériences, son milieu de prédilection, pour ne cultiver l'espèce que sur ce même milieu.

Restait à voir, si le procédé basé sur ces principes permettrait d'avancer, en évitant la confusion que l'on juge toujours menaçante, dès que la culture n'est pas irréprochablement pure. En renonçant à l'isolation mécanique, c'est donc sur l'effet du milieu que l'on compte, cet effet devant être assez prononcé pour provoquer un refoulement spontané des formes étrangères jusqu'à l'épuration de l'espèce que l'on vise.

Les cultures spontanées. Le procédé de ces cultures est employé principalement avec des espèces à fonctions spécifiques, mais aussi avec des espèces banales, ceci sous une forme quelque peu modifiée. Rappelons d'abord les formules de la série spécifique.

1 Les Nitrosobactéries. Gel silicique imprégné d'une faible solution de sulfate d'ammoniac et de sels nutritifs, émaillé d'une couche mince de carbonate de chaux ou de magnésie. Les colonies y apparaissent sous forme de plages de dissolution de l'enduit carbonaté.
2 Les Nitrobactéries. Gel imprégné d'une dose de nitrite de soude, émaillé d'une couche de poudre de Kaolin. Colonies minuscules, au-dessous d'un millimètre, glaireuses.
3 Ferments cellulosiques. Cytophagas et Cellvibrions. Gel imprégné d'une dose de nitrate de soude, couvert d'un rond de papier à filtrer, ou de morceaux de chiffons. Colonies sous forme de taches colorées de teinte vive — jaune d'œuf, orange, rouge, avec fibrolyse immédiate (*Cytophaga*); ou jaune terne et en nuances de brun, sans fibrolyse (Cellvibrions).
4 Fixateur aérobie. *Azotobacter*. Gel imprégné d'une faible dose de benzoate de soude, ou de calcium, et de sels minéraux, strictement dépourvu d'azote combiné.

5 Fixation anaérobie. *Clostridium*. Gel imprégné d'une dose de glucose, dépourvu d'azote assimilable. Plaques tenues dans une atmosphère d'azote pur.

L'éclosion de toutes ces colonies spécifiques, parfaitement caractéristiques et facilement identifiables, est assurée; bien entendu, si la terre ensemencée en contient les germes. Leur examen microscopique révèle un état de pureté satisfaisante, les germes étrangers ayant gardé leur état latent; dans quelques unes, on trouve encore quelque impureté que l'on élimine par repiquage. On ne repique que sur des plaques identiques, de même composition. Toutes les plaques ensemencées avec de la terre (le mieux, avec des grains minuscules déposés sur gel en raies régulières) donnent un tableau qui permet non seulement de constater la présence des germes spécifiques dans l'échantillon de terre, mais encore de juger en même temps, si la population spécifique y est dense ou rare et si elle n'est représentée que par une seule forme ou s'il y en a plusieurs.

De cette manière, on obtient ces organismes directement du milieu naturel à un état ne nécessitant plus aucune épuration préliminaire, pour être employé à des expériences destinées à l'étude de leur activité spécifique — Nitrification, Fixation etc., l'invasion des impuretés n'étant pas à craindre.

En choisissant, comme example, le cas de la Nitrification, comparons à ce procédé expéditif, l'ancien procédé datant de la fin du siècle dernier, utilisé après moi par tous les chercheurs, qui ont repris le sujet. On débutait par une série assez longue de cultures élective équivalente aux Anhäufungen de Beijerinck, et c'est de là que l'on cherchait à isoler le microbe spécifique par culture sur milieu solide, par dilution, ou par micromanipulation, laquelle est venue se joindre plus tard. Ces tentatives demandaient généralement de longs mois, en n'apportant souvent que des échecs. Le chercheur ayant probablement dépensé son énergie à cette besogne ingrate, n'arrivait plus à une étude plus compréhensive du phénomène dans la nature et du rôle de ses facteurs multiples. Il n'y avait toujours question que d'une seule espèce de Nitro- ou Nitrosomonade et c'est la grande difficulté de l'isoler à l'état de pureté complète et son comportement insolite envers les substance organiques, qui attiraient le plus d'interêt. Le travail fondamental de Kingma Boltjes, remarquable par sa technique, n'en fait pas exception. Il s'agit là toujours d'une seule forme admise identique à celle qui a été isolé en 1890, dont il a cru avoir isolé une seule

cellule en forme d'un petit coccus. Certes l'opération fait preuve de beaucoup d'adresse, mais la culture pure l'a empêché de noter l'existence de toute une florule nitrificatrice composée de formes bien différenciables par leur morphologie, leur oecologie et l'allure de leur activité spécifique.

Il est vrai que s'il s'agit spécialement du comportement de ces autotrophes envers les substances organiques, l'état de pureté absolue devient indispensable, mais il paraît alors logique de disjoindre cette question physiologique des études oecologiques sur le phénomène mondial de la nitrification.

Concernant la formule des plaques, destinées à la culture spontanée, l'emploi de la silice gélatineuse est de rigueur. On ne saurait la remplacer par de la gélose, car on n'y trouverait pas les avantages que présente ce gel minéral, libre de toute trace d'azote combiné; ce-qui permet de préparer des milieux de culture éminemment électifs et inattaquables par les moisissures. On ne saurait s'en passer notamment pour les expériences de fixation, lesquelles demandent un milieu bien libre de tout aliment azoté pour être conduites correctement, et qu'il est parfois nécessaire de prolonger durant de longs mois. Ce gel minéral a encore le grand avantage de se prêter très bien à la diffusion, de sorte qu'il est facilement imprégnable par toute les substances solubles, et qu'il se laisse „épuiser" aussi facilement qu'une solution aqueuse, même étalé en couche de 20 et 25 cm de diamètre. Enfin, pour l'analyse il n'y a qu'à sécher la plaque à douce chaleur, et y soumettre directement les quelques grammes de gravier silicique réduit en sable fin. Ajoutons enfin que les plaques à silico-gel sont beaucoup plus favorable à l'épuration par un processus de refoulement quasi naturel que les milieux gélosés.

Culture sur terre. L'examen microscopique directe du milieu terre fait partie de la nouvelle méthode; il est indispensable pour pouvoir employer la terre comme milieu de culture microbiologique.

Cette culture est particulièrement utile, lorsqu'il s'agit de formes caractéristiques, facilement reconnaissables à l'examen directe, telles que *Azotobacter*, *Clostridium*, baciles filamenteux, Champignons, Actinomycètes; tandis-que les formes petites ou délicates ne s'y prêtent pas bien (cocci, petits bâtonnets).

On peut cultiver les deux fixateurs dans la terre, qui les héberge, ou en les y ensemençant. C'est le procédé le plus sensible, pour

juger de leur présence, ou absence dans l'échantillon que l'on étudie.

Ils se laissent cultiver:
Les *Azotobacter*: 1° dans une couche de terre meuble légèrement tassée. 2° sur de la terre moulée en petite plaques; dans les deux cas, la terre est enrichie par une dose d'aliment hydrocarboné.
Les *Clostridium*: dans une terre empaquetée dans des tubes de 5 cm de diamètre et amenée à un dégré d'humidité convenable; la terre additionnée d'une dose de sucre.

Analyse microbiologique du sol. Hormis les groupes, à fonctions spécifiques, le sol est peuplé, comme on le sait, d'une foule de microbes actifs divers: petites formes de cocci et de petits bâtonnets courts, formes bacillaires sporogènes, champignons, actinomycètes, dont les fonctions se rapprochent ou font taches d'huile. Il ne serait pas possible de conduire l'étude de toutes ces formes hétérotrophes, sans pousser l'isolation jusqu'à la pureté controlée. Seulement, avant de les isoler, on cherche le milieu auquel elles sont adoptées, au-moyen de la culture spontanée.

Le procédé est décrit en détails (Ann. de l'Institut Pasteur **48,** 89,1932). Rappelons-le ici. On sème sur le gel imprégné ou enduit tour à tour, par des substances diverses, une dose déterminée, très faible de terre fine; on suit de près de jour en jour, d'heure en heure si nécessaire, l'éclosion des colonies, en s'appliquant à noter les plus précoces et les plus envahissantes; ce caractère étant un signe assez sûr que la souche est favorisée par l'aliment offert, jusqu'à dévancer et refouler les compétiteurs, apportés sur la plaque par la terre semée. En l'isolant incessamment sur un milieu de composition identique, on aura toute raison de s'attendre à ce que la souche y trouvera un milieu qui répond à ses adaptations.

Ce mode de selection des agents microbiens du sol pourra encore servir: 1° à éprouver la dynamique d'un sol donné, c'est à dire, le pouvoir de sa Microflora d'attaquer les substances offertes; 2° l'assimilabilité de certaines de ces substances pour un organisme ou un groupe donné; 3° le rôle des champignons et des Actinomycètes dans le sol arable; 4° les conditions de l'activité des fixateurs dans la nature.

Les formes nombreuses, qui paraissent sur les plaques spontanées au cours de l'analyse microbiologique, peuvent être isolées si

nécessaire en culture pure, d'autant plus facilement que la culture spontanée préalable aurait déjà fait connaître les aliments auxquels elles sont bien adaptées.

Discussion. Les recherches conduites d'après les principes qui viennent d'être exposés, ont fait comprendre que des études, limitées à la physiologie et à la biochemie des microorganismes, ne pourraient jamais nous éclairer sur le fonctionnement de la Microflore; ce fonctionnement ne devant pas être envisagé comme la somme des activités individuelles, mais comme le travail d'un collectif autoréglable. Il s'agit donc d'études foncièrement oecologiques; dont ceux qui maniaient la méthode classique n'ont tenu suffisamment compte. Autrement, ils auraient du s'abstenir de situer dans la libre nature le comportement des microbes, isolés au laboratoire en culture pure. Cependant, des conclusions qui prêtent à cette critique sont encore aujourd'hui énoncées couramment par les Microbiologistes, de sorte qu'une revue critique dirigée sur quelques examples, pourraît être utile, pour mieux préciser le point de vue de la nouvelle branche oecologique.

1 On sait que le nombre des espèces reputées ferments cellulosiques est très grand. On isolait une souche et aussitôt que l'on voyait une bande de papier noyée dans la solution aqueuse s'effilocher sous son action, on la croyait active au sein du sol. Or, des expériences de culture spontanée très nombreuses, ont montré que ce ne sont toujours que des Cytophagas et des Cellvibrions qui envahissent les fibres avec une rapidité prodigieuse, en les revêtant de manière à les préserver de l'attaque de microbes étrangers. Plus tard, ce sont des touffes, des champignons qui surgissent, en couvrant la plaque. Il paraît donc probable que ses ferments cellulosiques de laboratoire ne peuvent attaquer les fibres qu'en culture pure.

La spécificité des fonctions de ce groupe s'est imposée à tous les chercheurs; mais dans un travail récent Stanier croit devoir la nier, pour la raison d'avoir réussi la culture de *Cytophaga* sur glucose pure. Faudrait-il en conclure que l'espèce soit réellement capable d'attaquer le glucose au sein du sol, ou seulement potentiellement en culture pure? Pour chercher une réponse fondée sur l'expérience, le mieux serait, à notre avis, d'essayer des cultures spontanées, en déposant des grains de terre sur des plaques de silico-gel glucosé, pourvu d'azote nitrique. Je ne crois pas me tromper en affirmant que les plaques seront toujours

envahies par des bacilles et que les Cytophagas n'y paraîtront jamais. S'il en sera ainsi, on devra conclure que le glucose ne rend pas le milieu électif par rapport à ce groupe au sein du sol et que cette nutrition glucidique ne serait qu'une fonction potentielle, provoquée en culture pure, bref, un artefact physiologique. Ceci dit, bien entendu, sans préjudice pour l'intérêt biochimique qu'elle présente.

2°. Passons à un autre cas instructif, celui de l'*Azotobacter*. Depuis que Beijerinck l'a découvert, on se sert pour sa culture d'un milieu à dose relativement élevé de mannite ou glucose, additionné souvent d'azote nitrique, pour obtenir des cultures plus riches. Mais malgré sa culture facile et des recherches extrêmement nombreuses au cours de plusieurs dizaines d'années, la question de savoir, aux dépends de quelles substances aurait lieu la fixation dans la nature, restait obscure, le biotype étant incapable d'attaquer les matières, dont est composé le squelette végétal. D'autrepart, la culture sur glucides n'a pas manqué de modifier la morphologie de l'espèce, en provoquant la production excessive de formes aberrantes, sûrement anormales. Or, les expériences de culture spontanée ont montré que ces fixateurs ne paraissent pas sur des plaques à glucides, où ils se heurtent à des antagonistes puissants, mais qu'ils envahissent en maîtres absolus les milieux, qui ne leur offre que de mauvais aliments — tels que butyrate, benzoate, éthanol, butanol — à condition qu'ils soient dépourvu d'azote assimilable; car les antagonistes y sont réduits à l'impuissance. Ce sont donc ces deux conditions négatives — manque d'azote, mauvais aliments — qui déterminent l'état actif des fixateurs aérobies dans la nature. Quant à la morphologie aberrante, l'application du principe oecologique a fait voir, qu'elle est due à une sorte d'hypertrophie du biotype dans un milieu trop „riche" pour lui et qu'elle devient normale, à la suite de la culture dans un milieu „pauvre". En résumé, des deux méthodes ce n'est qu'au-moyen de la nouvelle, qu'il a été possible de dévoiler les conditions de l'activité des *Azotobacter* dans la nature.

3°. Les caractères physiologiques des autotrophes, ou inorgoxydants, ont été beaucoup discutés au cours de la cinquantaine d'années, écoulées depuis leur découverte; les études auraient montré que certains d'entre eux ne répondent pas aux caractères essentiels du type physiologique résumés dans nos recherches, à savoir: 1° végétation en milieu purement minéral, muni de la substance inorganique spécifique; 2° existence liée à la présence de cette

substance, qui subit une oxydation à la suite de leur processus vital; 3° ce processus étant la seule source d'énergie, dont ils disposent; 4° la substance organique n'est pas nécessaire ni pour la synthèse de leur substance, ni comme source d'énergie; 5° ils ne possèdent aucun pouvoir d'attaquer les substances organiques qu'ils ne tolèrent qu'à de très faibles concentrations; 6° seul, l'acide carbonique leur est indispensable, assimilé par chimiosynthèse.

Telle ou autre forme de ces autotrophes aurait été capable de végéter sans substance minérale spécifique, en utilisant l'aliment organique mis à sa disposition. Ces organismes ne seraient donc qu'un type physiologique mixte, leur autotrophie n'étant que facultative.

Il est à remarquer tout d'abord qu'aucun caractère physiologique n'est immuable dans la nature; qu'autour d'un type à caractères nettement tranchés on se heurte à des caractères attenués, instables, transitoires, en quelque sorte, vers un type différent. Ceci ne contredit nullement la réalité du type autotrophe f i x e que l'on trouve couramment dans la nature. On se demande plutôt, si le type modifié par la culture artificielle est capable de se maintenir dans le milieu biologiquement normal? De nouveau, on ne saurait admettre cette eventualité à moins qu'elle ne soit démontrée par des expériences spéciales. Jusqu'alors il est permis d'affirmer que les sulfobactéries, blanches et pourpres, ne sauraient se développer sans H_2S en dehors du laboratoire, tuées qu'elles seraient par le libre accès de l'air ni les v r a i s ferrobactéries sans protoxide de fer, ni les Nitrificateurs sans ammoniac etc. Et quant aux bactéries du gaz tonnant, il parait douteux que leur hétérotrophie puisse s'exercer face à l'activité énormement plus énergique des v r a i s hétérotrophes. En attendant, on ne saurait qu'admettre que les autotrophes restent fidèles à leurs fonctions dans la nature et que les modifications de fonctions qu'on leur fait subir ne sortent pas des confins du laboratoire, ne doivent donc être envisagés que comme des artefacts.

Voudrait-on interprêter cette hétérotrophie facultative, constatée au laboratoire, comme un caractère physiologique normal du groupe inorgoxydant, que l'on devrait appliquer la même qualification aux plantes vertes, cultivables, d'après des expériences déjà anciennes, sur solution sucrée à l'obscurité. Mais l'idée de les qualifier de hétérotrophes facultatifs n'est encore venue à personne, leur hétérotrophie n'étant possible en toute évidence que comme une expérience de laboratoire. Moins imposant chez les bactéries, le cas est logiquement pareil.

Je crois avoir démontré que l'application de la méthode classique à l'Oecologie microbienne des milieux naturels, spécialement le sol, ne saurait conduire qu'à des résultats inexacts, ou pour le moins incertains. C'est dire, qu'il serait bien temps de tracer une ligne de démarcation entre les études physiologiques et chimiques, d'un coté, et les recherches sur la dynamique microbienne des milieux naturels qui sont de nature oecologique. Cette disjonction rendraient les résultats réciproques plus nets, en écartant les malentendue et des critiques provoquées par un conflit de principes. Réellement, des cas de ce genre n'ont pas manqué d'arriver.

D'autre part, les origines et les débuts de la jeune branche étant en rapports étroits avec le développement des notions agrobiologiques et agrochimiques, il est évident qu'elle est appelée à servir la Science du Sol, en collaborant avec les disciplines qui y prennent part. Cette collaboration exigeant un grand nombre d'expériences, pour obtenir des résultats tangibles, nous avons tenu à rendre son procédé aussi expéditif que possible, en le débarrassant des opérations conventionnelles, qui prennent beaucoup de labeur, sans apporter de connaissances, qui en soient dignes.

Il y a une dizaine d'années mon ami L. G. Romell a bien voulu exposer essentiellement les mêmes idées, sur l'invitation du prof. A. J. Kluyver, au VI congrès de Botanique d'Amsterdam, publiées ensuite dans le Zentralbl. f. Bakt. II, **93**, 442, 1936 sous le titre „Winogradsky's quest of a Method in Soil Microbiology". Depuis ce temps, la méthode basée sur ces idées a subi quelques développements; mais la conclusion, si élégante et concise, de l'article anglais lui est toujours applicable. Je me fait un plaisir de la citer textuellement.

„Soil microbiology is not mere bacterial physiology; its main problems are ecological in nature; in the soil the organisms are specialized as to function due to natural competition. The competition factor cannot be replaced by any artifice of technique. A method for analyzing the microbial soil population according to true soil functions of the organisms, must work on this soil population as a whole. This is the principle of Winogradsky's direct method . . . . The method is not a substitute for pure culture methods, where such are needed, but a new developpement for an ecological purpose, which cannot be served by pure culture study".

(Department of Bacteriology, Iowa State College, Ames, Iowa. U.S.A.).

FIXATION OF HEAVY CARBON ACETALDEHYDE BY ACTIVE JUICES

by

NOEL H. GROSS and C. H. WERKMAN

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Introduction

No general agreement exists as to the mechanism of the biological formation of acetylmethylcarbinol. Evidence presented by Neuberg *et al.* (1918, 1920) supports acetaldehyde as an intermediate. Others have reported the formation of acetylmethylcarbinol from pyruvic acid (Dirscherl, 1930), acetic acid (Reynolds and Werkman, 1936) and citric acid (Hammer *et al.* 1935).

For a concise review of the early literature on the mechanism of the formation of acetylmethylcarbinol consult Stahly and Werkman (1942).

Silverman and Werkman (1941) were unable to show an increased yield of acetylmethylcarbinol, when acetaldehyde was added to a fermentation of pyruvic acid by their cell-free preparation of *Aerobacter*. They concluded that if acetaldehyde was necessary, their bacterial preparation did not possess the ability to activate the added aldehyde. Stahly and Werkman (1942) found the addition of acetaldehyde to a glucose medium resulted in an increased yield of acetylmethylcarbinol and 2,3-butylene glycol in fermentations by *Aerobacillus polymyxa* conducted either aerobically or anaerobically.

Green *et al.* (1942) have shown that the addition of acetaldehyde to pyruvate in the presence of a yeast juice and a juice obtained from pig heart gave an increase in the yield of acetylmethylcarbinol. This latter juice produced the carbinol from acetaldehyde alone.

Experimental evidence will be presented that will contribute to the clarification of the role of acetaldehyde as an intermediate in the formation of acetylmethylcarbinol.

Methods.

The bacterial enzyme preparations were made from 18–24 hour broth cultures of *Aerobacter indologenes* (23) and *Aerobacter aerogenes* (174) grown in glucose (1 %), Bactopeptone (0.3 %), $(NH_4)_2SO_4$ (0.3 %); K_2HPO_4 (0.5 %) tap water (10 %) medium. The juices were prepared by the glass grinding technic described by Silverman and Werkman (1941).

The yeast and animal enzyme preparations were obtained according to the method of Green *et al.* (1942).

The heavy carbon (C^{13}) acetaldehyde containing C^{13} in both carbon atoms was synthesized by the method of Cramer and Kistiakowsky (1941). The nitrogen used was passed over copper filings at 350° C., then through alkaline pyrogallate solution. O'Meara's (1931) test was employed in the detection of acetylmethylcarbinol. In some cases the Barritt (1936) test was used. Acetylmethylcarbinol was quantitatively determined according to Stahly and Werkman (1936).

Pyruvic acid was determined manometrically by ceric sulfate oxidation. At the conclusion of the experiment an aliquot of the acidified fermentation liquor was placed in a Warburg cup and 0.5 ml saturated ceric sulfate solution in N H_2SO_4 was placed in the side-arm. The contents of the side-arm were dumped into the main vessel after temperature equilibration. The reaction was continued for two hours with constant shaking in the water bath at 30.4° C.

Acetaldehyde was determined by the bisulfite titration of Donnally (1933) except that the bound bisulfite was liberated with K_2HPO_4 and titrated with 0.05 N iodine. K_2HPO_4 was employed because of interference of $NaHCO_3$ with the determination of C^{13}. Lactic acid was determined according to Friedemann and Graeser (1933).

Each compound to be analyzed for heavy carbon (C^{13}) was converted to CO_2. The CO_2 was then liberated from the alkali with 4 N lactic acid and C^{13} determined spectrometrically as described by Nier (1940).

The fermentation apparatus consisted of a chamber and three bead towers. The chamber consisted of a large pyrex tube stoppered with a three-hole rubber stopper. A piece of glass tubing, with a constricted orifice, dipped into the fermentation mixture to introduce the O_2 and CO_2 free nitrogen. Through the second hole was

placed a small water-cooled reflux condenser. In the third hole was a small piece of glass tubing fitted with a piece of rubber for the introduction of the reagents while keeping the system under anaerobic conditions. The series of bead towers was connected to the top of the reflux condenser. The first tower contained sodium bisulfite (2 %) to trap the aldehyde; the second contained $KMnO_4$ (half sat.) to trap any SO_2 and the third contained 1.5 N CO_2 free NaOH. The whole system was connected to a vacuum pump through a mercury trap, and a slightly reduced pressure maintained.

Bacterial Juice Fermentation.

The fermentation mixture consisted of: phosphate buffer, 1 M pH 5.4, 4.5 ml; Na pyruvate 0.3306 grams (3.0 mM); juice No. 2a, 2.5 ml; aldehyde (C^{13} in both carbons) 22.0 ml (2.27 mM); water 1.0 ml.

The pyruvate and the phosphate buffer were first placed in the fermentation chamber. CO_2 and O_2 free nitrogen was then passed through the liquid for fifteen minutes. Nitrogen was passed through the apparatus for another fifteen minutes and the other reagents added. Fermentation continued for 3.5 hours.

Four ml 6 N H_2SO_4 were added to precipitate the proteins, and the liquor was slowly brought to a boil. The entire system was then flushed for 30 minutes to carry over the CO_2 and the acetaldehyde.

Aliquots of the mixture were made up to 50 ml, filtered and used to (1) test for residual pyruvate, (2) total carbon, (3) isolation and purification of the acetylmethylcarbinol.

Yeast Juice Fermentation.

The fermentation mixture consisted of: phosphate buffer, 0.5 M, pH 7.6, 1 ml; enzyme preparation, 15 ml; Na pyruvate, 0.2478 g (2.25 mM); acetaldehyde (C^{13}) .1014 M 15 ml (1.52 mM); water (CO_2 free) 3 ml (Total 34 ml).

The enzyme preparation was added to the fermentation tube and the system connected to the first bead tower was flushed with O_2 free and CO_2 free nitrogen. The sodium pyruvate prepared according to the method of PETERS (1938) was weighed directly into the acetaldehyde. After complete anaerobiosis had been established and the other reagents added, the temperature was kept at 38° C. in a water bath. The fermentation was allowed to continue for two hours and twenty minutes, when 6 ml 6 N H_2SO_4

were added and the mixture slowly brought to a boil. A slightly reduced pressure was maintained throughout the experiment. After the fermentation had come to a boil, nitrogen was gently passed through for 30 minutes to remove the acetaldehyde which was collected in 50 ml of a 2 % bisulfite solution, and made up to 100 ml. The CO_2 was collected in 10 ml 1.5 N CO_2 free NaOH solution made up to 100 ml.

Pig Heart Fermentation.

The activity of the pig heart preparation was determined manometrically. Each cup on the Barcroft-Warburg respirometer contained 1 ml of the enzyme preparation, 0.1 ml diphosphothiamin (150 ml), 0.1 ml $MgSO_4$ (0.1 %), substrate and enough water to make a total volume of 2 ml. Anaerobiosis was obtained by flushing the cups with nitrogen.

Experimental.

The results (Table I) indicate that acetaldehyde is not an intermediate in the formation of the carbinol by the bacterial juice. The concentration of C^{13} in the added acetaldehyde was 2.27 mM, whereas that of the recovered aldehyde was 2.14 mM, a decrease of 0.13 mM. Of the 3.0 mM of sodium pyruvate added, 2.45 mM were dissimilated and 1.04 mM of acetylmethylcarbinol formed.

Table I.

C^{13} Acetaldehyde Fixation by *Aerobacter aerogenes* Juice.

Compound	Millimoles	Excess C^{13}
Aldehyde added	2.27	3.16
Aldehyde recovered . . .	2.14	3.04
Carbon dioxide	—	0.00
Fermentation liquor . . .	—	0.03
Acetylmethylcarbinol . .	1.04	0.00
Pyruvate fermented . . .	2.45	—

The original acetaldehyde contained 3.16 atom per cent excess C^{13}. The recovered acetaldehyde contained 3.04 atom per cent excess C^{13}, a difference which may be experimental. The CO_2 contained no excess C^{13}.

With the yeast juice 1.52 mM of heavy carbon acetaldehyde

Table II.

Fixation of Heavy Carbon Acetaldehyde by Yeast Juice.

Compound	First Fermentation		Second Fermentation	
	mM	Excess C^{13}	mM	Excess C^{13}
Pyruvate fermented . . .	1.84	—	2.91	—
Acetaldehyde (C^{13}) added.	1.52	3.16	3.27	3.67
Acetaldehyde recovered .	2.28	0.88	3.76	2.28
Carbon dioxide	1.82	0.00	2.83	0.00
Acetylmethylcarbinol. . .	0.32	0.88	0.52	1.44
Fermentation liquor . . .	—	0.32	—	0.30

were added to the first fermentation (Table II) and 2.28 mM of aldehyde recovered. Thus considerable dilution of the added aldehyde resulted as evidenced by the excess C^{13} in the recovered aldehyde (0.88 %), whereas the original aldehyde contained 3.16 per cent. There were 0.325 mM of acetylmethylcarbinol formed with an excess C^{13} percentage of 0.88 which is substantially higher than the normal abundance and indicates that heavy carbon acetaldehyde entered into the formation of the acetylmethylcarbinol.

The excess C^{13} content of the fermentation liquor was 0.32 per cent and showed that a considerable amount of acetaldehyde had been transformed into some compound that was not removed by flushing with nitrogen.

There were 1.84 mM of sodium pyruvate fermented by this yeast juice. For each millimole of pyruvate fermented one of CO_2 was formed. The CO_2 contained no increase in C^{13} content and thus did not originate in the added acetaldehyde.

In the second fermentation 3.27 mM of heavy carbon acetaldehyde were used and 3.76 mM were recovered, an increase of 0.49 mM. This increase, although not as large as in the first experiment, does indicate formation of acetaldehyde from the pyruvic acid. This biologically formed acetaldehyde diluted the excess C^{13} content of the added aldehyde. The original aldehyde contained 3.67 per cent excess C^{13}, whereas the recovered aldehyde contained 2.28 per cent.

There was approximately one millimole of CO_2 formed for each millimole of pyruvate fermented in each fermentation. The C^{13} of the CO_2 was normal.

The increase in C^{13} content of the fermentation liquor was approximately the same as in the first experiment.

With pig heart juice 16 μl of CO_2 were evolved during the first ten minutes from pyruvate (Table III). During the same period

Table III.

CO_2 Production from Pyruvate, Acetaldehyde, and Pyruvate + Acetaldehyde by Juice from Pig Heart.

Minutes	Pyruvate .03M	Acetaldehyde .036M	Pyruvate .03M plus Acetaldehyde .036M
	mm^3 CO_2	mm^3 CO_2	mm^3 CO_2
10	16	0	38
165	60	0	42
280	66	—2	40
A.M.C.	++	++++	++++

there were 38 μl of CO_2 produced from the pyruvate plus acetaldehyde. The presence of acetaldehyde increased the initial rate of CO_2 production. The total CO_2 formed from pyruvate plus acetaldehyde, however, was considerably less than from the pyruvate alone and was much less than that from either the bacterial or the yeast juice.

No CO_2 was formed from the acetaldehyde during the entire experiment with pig heart juice.

The addition of acetaldehyde to pyruvate appreciably increased the formation of carbinol; however, there was as much acetylmethylcarbinol formed from the acetaldehyde alone as from the acetaldehyde plus pyruvate. The total amounts of acetylmethylcarbinol as evidenced by the O'Meara creatine test were much greater than that formed by either the bacterial or the yeast juice. Large amounts of acetylmethylcarbinol with no large quantities of CO_2 were formed; from acetaldehyde no CO_2 was evolved.

Discussion and Summary.

Juices prepared from *Aerobacter* did not utilize synthetic acetaldehyde in the formation of acetylmethylcarbinol. Heavy carbon

acetaldehyde when added to a pyruvic fermentation yielded acetylmethylcarbinol with only the normal abundance of heavy carbon. The added C^{13} acetaldehyde was recovered with only a slight reduction in heavy carbon content. The amount of acetaldehyde recovered was just slightly less than that added to the fermentation. If acetaldehyde had been formed in the breakdown of the pyruvic acid then there should have been a marked dilution in the heavy carbon content of the recovered acetaldehyde. This suggests that some other compound serves as an intermediate, possibly a phosphorylated compound. LIPMANN (1939) indicated that in the breakdown of pyruvate by *Lactobacillus delbrückii* a phosphorylated intermediate occurs.

The results confirm the observations of SILVERMAN and WERKMAN (1941).

The yeast juice enzyme preparation, on the other hand, utilizes synthetic acetaldehyde to form acetylmethylcarbinol from pyruvate. When heavy carbon enriched acetaldehyde was added to the yeast juice fermentation the acetylmethylcarbinol contained an increased amount of heavy carbon, thus confirming the investigations of GREEN *et al.* (1942).

The amount of acetaldehyde recovered from the yeast juice fermentation was greater than the amount added and the percentage of heavy carbon was decreased, all of which suggests the dissimilation of pyruvic acid to acetaldehyde. The amount of carbon dioxide formed was practically the same as the amount of pyruvate fermented which indicates a decarboxylation of the pyruvate to CO_2 and acetaldehyde. The yeast juice preparation is, therefore, quite different from the bacterial preparation.

The pig heart preparation is different from either the yeast or the bacterial juice in that it can produce acetylmethylcarbinol from acetaldehyde and in large quantities. Diphosphothiamin (GREEN, 1942) was essential for the condensation of the acetaldehyde to acetylmethylcarbinol. The pig heart preparation differs from the bacterial and the yeast preparations in that considerable amounts of acetylmethylcarbinol are formed from pyruvic acid with no appreciable formation of CO_2.

Do these results mean that there are three mechanisms of acetylmethylcarbinol formation or that each preparation contains enzyme systems capable of activating certain substances that ultimately are transformed into the intermediates? That is, does the pig heart juice have enzymes than can form the intermediate directly from

the synthetic acetaldehyde, perhaps a very strong phosphorylating mechanism, which the yeast and bacterial preparations do not have? The yeast preparation, on the other hand, may be able to combine pyruvic acid and acetaldehyde, as suggested by DIRSCHERL (1931) before decarboxylation, while the bacterial preparation does not have any enzymes that can activate synthetic acetaldehyde, but can form the intermediate from the pyruvate. These are questions that await further investigation.

The bacterial enzyme preparations obtained from *Aerobacter* did not fix heavy carbon acetaldehyde in forming acetylmethylcarbinol.

The yeast juices prepared from air-dried yeast did fix heavy carbon acetaldehyde in forming acetylmethylcarbinol in the presence of pyruvate.

The pig heart enzyme preparation formed acetylmethylcarbinol from acetaldehyde alone.

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(Institute vir Siektekunde, University of Pretoria, South-Africa).

FILMING AS A METHOD OF RESEARCH IN MICROBIOLOGY

by

ADRIANUS PIJPER

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Microbiology can be approached in a variety of ways. Some of these at times appear exhausted, and then are dropped, to be replaced by others which appear of greater promise. The present time witnesses an emphasis on biochemical approach, and this has enjoyed the consistent attention of Professor A. J. KLUYVER and his school. Brilliant work has been done by this school under its enthusiastic leader. In the medical section of microbiology this approach is gaining attention, but is hampered by inadequate chemical training of workers, and by the traditional tendency towards serological differentiation, to which chemical analysis is not readily applicable.

Bacterial taxonomy has never been quite satisfactory, witness the numerous „systems" that have been advocated. By most workers morphology is still put first and foremost, and biochemistry or physiology second, whenever classifications of bacteria are discussed. There is no doubt however that biochemistry is gaining ground, somewhat to the detriment of morphology. It is usual to complain of paucity and scarcity of morphological criteria. Biochemical researches on bacteria have much more to offer, as beautifully illustrated by the researches of Prof. KLUYVER and his school. These researches took various directions, and although they were not undertaken for taxonomic purposes, it is unavoidable that they find application in taxonomy.

The emphasis on biochemistry seems to me to be at least partly due to the idea that morphology is played out and has nothing further to offer. Microscopy of bacteria has got into a backseat. When it is still practised, it uses staining processes with a distinct biochemical basis, it has become micro-chemistry. Considering all these matters from the viewpoint of a traditional medical bac-

teriologist, I have no fault to find. Quite the contrary, I feel sure that the newer biochemistry of bacteria will assist medical pathology in many of its problems, and as regards improved micro-chemical staining methods for microbes, I have no doubt that here too microbiology stands to gain.

But notwithstanding all this, I still believe that there is room for a purely morphological approach, based on the study of bacteria with a microscope. My contention however is that such studies should be undertaken on living material. I have a feeling that microbiology and especially bacteriology has rather neglected looking at bacteria in the live state. If I may bring up a parallel case, human pathology started in the post-mortem room. But the time is past that human pathologists are satisfied with the study of the dead body. They have advanced beyond that, with great benefit to their science.

Nowadays pathologists pay much more attention to live material than to the dead body, they are more interested in the movement of things than in the days when a pathologist was synonomous with a pathological anatomist. Bacteriologists have spent many years looking at dead bacteria. Is it not time they devote at least some of their energy to the analysis of the conduct of live bacteria under the microscope? It may mean the introduction of new techniques, or at least the improvement of existing techniques. Amongst these I would place foremost the employment of dark-ground methods, combined with a film camera. I imagine that my own work on these lines has thrown new light on the process of agglutination.

It would take too long to go into the details of these techniques, or as I would rather call them, methods of investigation or research. The efficiency of darkground microscopy stands in direct proportion to the brilliancy of the lightsource, and so one has to fall back on the sun. No electric lightsource so far designed, can rival the sun. For scientific purposes the 16 mm film is very suitable, the 8 mm variety has too small a field, and the 35 mm variety requires too bulky an outfit for convenient use with a microscope. I look forward to the day when an enterprising firm will place on the market a special small size 16 mm film camera that can be directly attached to a microscope, to take the place of the present ones which still need a support of their own. The miniature camera has largely replaced the bulky photomicrographical outfit commonly used in former days for still photography, and

there is no reason why film cameras should not follow suit.

To most biologists a film is still just a method of illustration, and very little used at that. I have always maintained that there is a good deal more in filming than that, and my own experience, on which I am going to draw in the following pages, has vindicated this. To me, filming microbes has become a method of study and research.

To begin with, when one sets about to film any kind of activity of microbes, one has to start by „clearing the decks". One has to clear away all accessory or incidental happenings and effects. It is the need for clarity in the film, which necessitates clarity in the microscopic field, which in its turn brings about a higher degree of clarity of thought as to what one is after. It has been said that a thing or a happening is only clear to one when one can make a model of it. Nowadays one achieves the same by making a film of it. Of course one cannot always film everything that is there, especially when one reaches higher magnifications with the microscope. It is eminently regrettable that the makers of film material have not yet succeeded in supplying photographic emulsions that will record everything than the eye can see. How often have I deeply regretted that very important microbiological phenomena which were so obvious to the eye, had to be left unrecorded, simply through the lack of sensitiveness of the film material. But this does not diminish the value of what can be recorded. A film record of a phenomenon is so much more valuable than a written record. A written record of an observation is nothing but a petrified impression which a happening has made on the observers usually somewhat prejudiced mind. It may have nothing to do with what actually happened. The camera does not tire, as our eye does, sometimes at a critical moment. It is not prejudiced, as we mortals always are. Subconsciously we may adjust our so-called observations to what was in our mind before we started, most of us see chiefly what we expect to see. And the phenomenon which we have witnessed and of which we have written down our interpretation, for that is all we can do, that identical phenomenon cannot be repeated, except by a film. I have found it astounding how films I had made years ago, when projected again, took on quite a different significance. The phenomenon as recorded had not changed but my insight into it had ripened. One can to a certain extent manipulate or „fake" still-photographs. A still-photograph only represents one aspect of a thing at one moment at one spot.

It is not so with a film, because the film extends our objective records into time. And the object through its very motility changes its milieu and localisation.

A further advantage of cinemicrography as a research method is that whilst ordinarily only one person at a time can view a happening under a microscope, any number of persons can observe, study and comment upon such a happening when it has been filmed and is projected. It can be discussed, and this often is of benefit to the original observer.

One technical point still merits attention. Running a film through the projector backwards, makes it possible to see events reversed in time. I can see a bacterium which through bacteriophage action has degenerated into an unrecognisable mass or has exploded into nothingness, reintegrate and become a normal bacterium again, simply by showing the film in reverse.

Whilst writing the above, I had chiefly in mind what is called „straight filming". This means pictures both made and projected at identical speed, usually 16 pictures per second, this being the minimum speed necessary to create the impression of continuous movement. But the same considerations apply to other techniques.

Apart from „straight filming" there is „time-lapse filming" and „slow motion filming". In the latter two methods one can play with time, slowing down a microscopic happening, or speeding it up, as circumstances may require. The „time-lapse" method is particularly suitable for slow processes, such as growth. The „slow motion" method is applicable to events which naturally take place at such high speed that they cannot be followed and certainly not analysed by the human eye. It is literally true that either method makes visible to the eye what no human eye could ever see by itself. My own work has greatly benefited from either method.

We all believe that bacteria multiply usually by splitting. But how many of us have seen them doing it? It was quite a revelation to me when I saw it happen on one of my films. It is a difficult subject to film, for various reasons. The objects being small, one needs a very brilliant light, and the necessarily long exposures interfere with the life of the bacteria. Here progress, and better insight into cell-division is very much bound up with improvements in technique. But even now, with imperfect technique, it is worthwhile to put up microscope and film camera, activate the camera by some kind of clockwork so that an exposure is made say every quarter of a minuet, then leave the whole apparatus to itself for

hours at a stretch, and then to see bacteria split, separate and slip past one another in the finished film when projected. Again, we all believe that yeastcells multiply by budding. But how few of us have ever sat it out to see it happening? My film shows me nine yeast cells all being derived from one original cell, and it does so in a few minutes, whilst the actual process and filming time occupied several hours. I need not tell my students now that yeasts bud, they can see it with their own eyes. But there is more to it. It happened that one spectator one day pointed out to me a feature of this bit of film that had escaped me. Nearly every time a yeastcell started blowing the initial bubble from its cellwall which was going to be a new yeastcell, the wall exactly opposite began to change its shape. It became pushed out slightly as if the wall gave way to increased localised pressure. It looked as if the cell was straining itself to produce the bud, and that the strain went right across the cell and bulged the opposite cellwall. I do not know what it really signifies, but it does happen, and it is very curious to watch, but it would never have been seen without filming.

Coming back to „straight filming" it is many years now since I first demonstrated, by means of a film, that motile bacteria when they swim straight, show a long thin tail. The film would probably not have been made if I had not been anxious to demonstrate this tail, which at the time I looked upon as a motor organ. But at the same time, my prolonged occupation with this tail, which became necessary for its proper demonstration in a film, led to further study of this curious appendage, with unexpected results. The tail is extremely thin, and requires very brilliant light to become visible. Under such conditions the bacterial body becomes just a blur, as it radiates too much light, partly because it is bigger but also because it moves so fast. This circumstance made me miss the essential feature of bacterial movement, another example of that one only sees what one looks for. When on the advice of Professor R. Breed I plunged my high-speed bacteria into colloid methylcellulose solutions, speed was slowed down very considerably (the explanation of this is not just the obvious one), and the true nature of bacterial motility revealed itself. The high-speed bacteria, reduced to much lower speed, became a mass of gyrating undulating bodies, evidently propelling themselves through the contortions of their cell-walls, and dragging spiral tails behind them. The gyrating undulating movement was obvious both in the longer and in the smaller forms. Micro-organisms that have been going under the

name of bacteria, which means ,,rods", now presented their true shape, which was that of a spirillum, which means a ,,coil". The name ,,bacteria" was bestowed upon them because they were chiefly studied as corpses, in full *rigor mortis*, beautifully preserved, embalmed and even painted up. It had now turned out to be a misnomer, and this is what I meant when I said at the beginning that microbiology should watch living microbes.

This new conception of ,,bacteria" really being spirals required confirmation. I went back to a ,,slow motion" film of the same bacteria under normal conditions made previously and then put aside, as not of much interest. I had made the camera take pictures at the rate of 64 per second, and by projecting at 16 pictures a second, the bacteria appeared to move four times slower than they actually did. The pictures were rather faint, on account of the shorter exposures, but this had the advantage that the outlines of the bacterial bodies were clear-cut, instead of blurred. And now I saw, what I had overlooked at previous showings, that here also the ,,bacteria" moved as spirals.

My conviction that motile ,,bacteria" are not ,,bacteria" but should be called spirillums thus became greatly strengthened. Elsewhere I have brought together further observations and considerations in this connection. Here I shall just devote a few more words to the problem of flagella. I have shown previously that what I have filmed as a tail, is a mass of wavy threads, wound together. Under certain conditions they become unwound. This may happen in a drying drop on a microscope slide, and they can then sometimes be stained to appear as a tangled mass of wavy threads, which are called flagella. The electron microscope can show them up without staining. They are usually regarded as motor organs. In reality they are the product of motility. The undulating gyrating movement of the bacterial body twists the polysaccharide covering of motile bacteria into wavy threads, which during forward movement combine into a tail.

This is what filming motile bacteria has brought to light. It can be put in very few words now, but it has been a long process, with many false routes, and much retracing of steps. But the whole process is a good illustration of the filming method of research. Filming disciplines observation, and necessitates working under strictly experimental conditions, excluding everything that is immaterial. Conversely, the evidence collected from comparing all the completed films (and many more were made than are mentioned

here) allowed of one conclusion only, and that was that motile bacteria move by means of a gyrating undulating movement of their bodies, and that tails and flagella are merely secondary appendages, and not motor organs. This is a far reaching conclusion, defying the shades of COHN, KOCH and LOEFFLER, and its implications comprise the whole of bacterial classification. Morphology has been overshadowed by biochemistry for a long time, it is now getting its own back. If the biochemists accept the new position, morphology can afford to make them a present of the polysaccharide twirls, which used to go under the name of „flagella", for chemical analysis. Bacterial specificity resides largely on the bacterial surface, and the surface covering has already been the subject of chemical analysis. „Flagella" have now become surface material in a special configuration, nothing more, and the biochemists can have them. I hope they will accept them in the spirit in which they are offered, the spirit of co-operation. We shall only be able to understand bacteria properly if we work together, and not in watertight compartments. That is what I have tried to do, and of which I have given an example in the preceding pages. Morphology can assist biochemistry and biochemistry must come to the aid of morphology. Bacteriologists will look forward to the results of chemical analysis of the appendages that used to be regarded as „flagella" and which now as the result of a new conception stand revealed as just twisted surface material. But I would like it to be remembered that this new condition owes its origin to cinemicrography, to the use of a film camera attached to a microscope.

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(From the Medical Research Council Unit for Chemical Microbiology, The Biochemical Laboratory, Cambridge, England).

SOME ASPECTS OF HYDROGEN TRANSFER

by

MARJORY STEPHENSON

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> „Si l'on venait de me dire que dans ces conclusions je vais au delà des faits, je répondrais que cela est vrai, en ce sens que je me place franchement dans un ordre des idées qui, pour parler rigoureusement, ne peuvent être irréfutablement démontrées. Voici ma manière de voir. Toutes les fois qu'un chimiste s'occupera de ces mystérieux phénomènes, et qu'il aura le bonheur de leur faire un pas important, il sera instinctivement porté à placer leur cause première dans un ordre de réactions avec les résultats généraux de ses propres recherches. C'est la marche logique de l'esprit humain . . . ."
>
> Louis Pasteur, Ann. Chim. Phys. 3e Sér. **52**, 404, 1858; also Oeuvres **2**, 13, 1922.

The conception of biological oxidation as taking place by successive transfers of paired hydrogen atoms to an acceptor or reducible substance has become a commonplace. First put forward by Wieland and developed by Thunberg it was introduced into microbiological chemistry by Kluyver who used it as a basis for a unified conception of the intermediary reactions by which, under the influence of microbial action, carbohydrate is transformed in the varied processes of fermentation (22). Under his inspiring guidance there emerged from the Delft School during the late twenties and early thirties a series of biochemical studies comprising all the principal bacterial fermentations familiarising bacterial chemists with the view that, although the final products are so diverse, yet many of the same fundamental processes are at work in all. Certainly our views concerning the details of these processes have undergone many changes since those days but the conception of a unified pattern of events underlying very diverse

phenomena was first inspired by KLUYVER's teaching. Moreover — and most important — so ably and thoroughly was the analytical work carried out that this series of theses still remains the most reliable source of quantitative data to which workers on bacterial fermentations may turn.

Now although it is true that many fundamental chemical reactions are common to animals plants and microbes it is easy to overestimate the unity of nature when bacteria are being considered in relation to animals and plants. It is probably true to say that whilst most chemical changes occurring in the latter are either duplicated or closely parallelled in some microorganism or other yet the converse is far from being true. The number and variety of chemical reactions already known to be catalised by bacteria far exceed those attributable to the animal and the plant together. Moreover amongst heterotrophs it is as anaerobes that bacteria specially excel; this implies that this group has elaborated special enzymes for oxidising their substrate molecules without having recourse to oxygen; in other words it is in the use of hydrogen acceptors that bacteria are specially developed as compared with animals and plants.

The Metabolism of Hydrogen.

The oxidation of hydrogen was first observed in that class of autotrophs (Knallgasbakterien) which use the energy thus obtained for the reduction of CO_2 and hence for the synthesis of cell substance. These early classical studies (21, 31) were physiological in nature and were not concerned with the underlying biochemical mechanisms; indeed they were made before the development of enzyme studies had begun.

For a long period the oxidation of molecular hydrogen was believed to be confined to these autotrophs; it was not until 1931 that it was shown to be much more widespread and not confined to organisms utilising the reaction as its sole source of energy for growth (33). It is in fact the property of a large number of anaerobes and facultative anaerobes to catalise the reaction $H_2 + A \rightarrow AH_2$, that is to reduce compounds by means of molecular hydrogen. The enzyme catalysing this reaction has not so far been reported as having been prepared in a state of purity, but it has been obtained in a cell free condition from crushed or ground bacteria (20, 23).

Function of hydrogenase.

The „Knallgas bacteria" must obviously possess some mechanism for linking the energy liberated in the oxidation of hydrogen to the synthetic processes of the cell; the nature of that mechanism is at present unknown; one might perhaps explore the possibility of hydrogen oxidation being linked with phosphorylation processes culminating in the production of adenosine triphosphate on the analogy of the oxidation of sulphur by the autotroph *Thiobacillus thiooxidans* (37). Be this as it may the function of hydrogenase amongst heterotrophs has yet to be determined. Is the oxidation of hydrogen here linked with some synthetic process contributing to cell synthesis? Or does it play some other part or is it, in the case of the heterotrophs, a superfluous mechanism readily dispensed with? One fact may be noticed; the reversible system $H_2 \leftrightarrows 2\,H$ has the highest reduction potential of any biological system ($rH = 0.0$; E_0 at pH 7.0 and 30° C. = —0.445 volts); hence it is able — provided the enzyme activating the acceptor is present — to keep reduced a larger range of oxidisable compounds than any other reducing agent, not excluding cystine – cysteine and other –SH compounds. Now enzyme studies on cell free systems frequently show that enzymes lose their activity rapidly on becoming oxidised, so hydrogen may well play the part of an intracellular reducing agent; indeed evidence for this function has been gained in the preservation of serine deaminase (14).

The Use of Hydrogenase in Biochemical Studies

But whatever may be the function of the hydrogen-hydrogenase system in the cell it is a remarkably useful tool in the hands of the bacterial chemist, for by its use bacterial reductions can be studied rapidly and quantitatively by manometric methods and the products of reduction obtained unmixed with products of oxidation; in fact hydrogen transfer is offered, as it were, on a place in its simplest possible form. A series of problems clarified by this means will now be considered.

Reduction of nitrate.

The reduction of NO_3' to NO_2' has long been a diagnostic test in the identification of bacteria; the enzyme catalysing this reaction was first studied by Stickland (34), but the reduction of nitrate by hydrogen, using *Cl. welchii*, was investigated exhaustively by

Woods (40) by the manometric technique. The course of the reaction is shown in fig. 1. Here four reactions are displayed: first a fast reaction in which 1 mol. H_2 per mol. nitrate disappears; this is followed by a slower reaction till 4 mols hydrogen have disappeared; at this stage nitrite is no longer present and has given place to ammonia; this suggests that nitrite can be reduced to ammonia as is shown on the second curve. The probability that nitrite is reduced to ammonia via hydroxylamine is confirmed by the reduction of the latter, shown on the third curve.

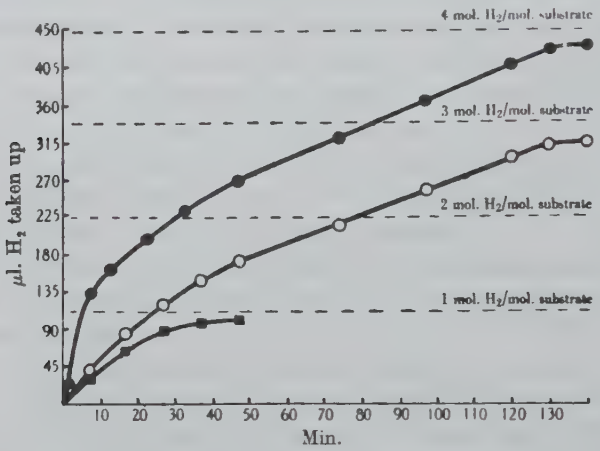


Fig. 1. Course of H_2 uptake. • nitrate, o nitrite, ▪ hydroxylamine. 1 ml bacterial suspension (6,2 mg/ml), 1 ml $M/5$ phosphate buffer pH 7,1, 0,1 ml $M/20$ substrate, 0,4 ml water. From Biochem. J. **32**, 2002, 1938.

The following quantitative reactions were proved to occur:

$$HNO_3 + H_2 = HNO_2 + H_2O \quad -\triangle H = 50 \text{ Cals.} \tag{1}$$

$$HNO_3 + 4\,H_2 = NH_3 + 3\,H_2O \quad -\triangle H = 167 \text{ Cals.} \tag{2}$$

$$HNO_2 + 3\,H_2 = NH_3 + 3\,H_2O \quad -\triangle H = 185 \text{ Cals.} \tag{3}$$

$$NH_2OH + H_2 = NH_3 + H_2O \quad -\triangle H = 55 \text{ Cals.} \tag{4}$$

The heat liberated in these reactions is shown; whether the energy so made available is utilised by the organism, as in the case of the reduction of nitrate by organic hydrogen donators, is unknown.

Sulphate Reduction.

The study of sulphate reduction in its fundamental aspects belongs to Holland (10, 11); it was studied in detail by Baars (1) in 1930 who showed that a number of hydrogen donators could be used for the reduction, these depending on the strain employed. In 1931 a strain was found (33) using molecular hydrogen and the reaction shown to conform quantitatively to the equation

$$Na_2SO_4 + 4\,H_2 = Na_2S + 4\,H_2O \qquad (5)$$

Hydrogenase is by no means universally found in this group but when present accounts for the appearance of hydrogen sulphide in coal gas, and in situations where organic material is absent or at a minimum.

Reduction of CO_2. (1) methane production.

Probably CO_2 is the most important oxidising agent amongst anaerobes, and it is only recently that its function in this respect has become apparent. Its part as hydrogen acceptor in bacteriological transfers of hydrogen was first recognised by Kluyver (22) who saw in methane the most reduced product in the fermentative chain. Early work on methane associated its production with cellulose fermentation; it is now clear that this association was fortuitous and due to the fact that cellulose fermenters and methane producers occur together in many situations, such as the gut of herbivores, marshes, bogs and river bottoms, and that both types are somewhat difficult to get into pure culture. The first clarifying observation in this field was due to Söhngen (32) who showed that in the presence of enrichment cultures of a methane producer the following reaction took place

$$\begin{gathered} CO_2 + 4\,H_2 = CH_4 + 2\,H_2O \\ -\!\!+ H = 59 \text{ Cals.} \end{gathered} \qquad (6)$$

This revealing observation should have put bacterial chemists immediately on the right track; but actually this key observation was not extended for 30 years, till Barker, in a recent series of masterly experiments, cleared the whole field of methane production (4, 5).

Söhngen's may be regarded as the key reaction of which

$$4\,H_2A + CO_2 = 4\,A + CH_4 + 2\,H_2O \qquad (7)$$

is the generalised form.

Various strains of methane producers have been isolated in-

cluding one which brings about the Söhngen reaction, appropriately named *Methanobacterium Söhngenii*. In general fatty acids are oxidised to CO_2, primary alcohols to fatty acids and secondary alcohols to ketones.

$$2\,C_2H_5OH + CO_2 = CH_4 + 2\,CH_3.COOH \quad -\triangle H = 22 \text{ Cals.} \tag{8}$$

$$2\,C_4H_9OH + CO_2 = CH_4 + 2\,C_3H_7COOH \quad -\triangle H = 16 \text{ Cals.} \tag{9}$$

$$4\,CH_3.CHOH.CH_3 + CO_2 = CH_4 + 4\,CH_3.CO.CH_3 \quad -\triangle H = 22 \text{ Cals.} \tag{10}$$

Radioactive carbon has been used to gain further insight into this reaction; washed cell suspensions were incubated with radioactive C^*O_2 and ethanol (reaction 8); at the end of the experiment CO_2 was prepared from the cells, the methane and the acetic acid; radioactive carbon was found in the methane and in the cells but not in the acetic acid proving that methane arises by the reduction of CO_2; it is also interesting as showing that reduced CO_2 contributes to the cell material and that the oxidation of ethanol is coupled with some reduction of CO_2 used in cell synthesis (Barker *et al.*).

Reduction of CO_2 (2). Acetic acid production. In 1936 Wieringa (43) found that canal mud inoculated into inorganic medium with H_2S and $NaHCO_3$ absorbed hydrogen rapidly. He pasteurised the culture and obtained a pure strain of a *Clostridium* by plating. This organism, inoculated into the original medium, again took up hydrogen rapidly forming acetic acid; the reaction was shown to occur quantitatively.

$$4\,H_2 + 2\,CO_2 = CH_3.COOH + 2\,H_2O \quad -\triangle H = 59 \text{ Cals.} \tag{11}$$

This then is a new type of CO_2 reduction and the conditions of the experiment leave little doubt that here the organism is using the energy obtained by this reaction for cell synthesis.

A knowledge of the Wieringa reaction has led to the understanding of several bacterial processes which must otherwise have presented insoluble problems. Two anaerobes, *Cl. acidi urici* and *Cl. cylindrosporum*, isolated from soil by Barker and Beck (7), were found to develop poorly on yeast water and other ordinary laboratory media commonly used for Clostridia but to grow readily on a synthetic medium containing uric acid in presence of sodium

sulphide. Washed suspensions decompose uric acid readily, also xanthine, hypoxanthine and (less readily) guanine. The four purines are attacked rapidly and completely, the only products being acetic acid, CO_2 and NH_3.

$$C_5N_4H_4O_3 + 5.5\ H_2O = 4\ NH_3 + 3.5\ CO_2 + 0.75\ CH_3COOH \quad (12)$$
uric acid $\qquad -\triangle H = 259$ Cals.

$$C_5N_4H_4O_2 + 6\ H_2O = 4\ NH_3 + 3\ CO_2 + CH_3COOH \quad (13)$$
xanthine

$$C_5N_4H_4O + 6.5\ H_2O = 4\ NH_3 + 2.5\ CO_2 + 1.25\ CH_3.COOH \quad (14)$$
hypoxanthine

$$C_5N_5H_5O + 7\ H_2O = 5\ NH_3 + 3\ CO_2 + CH_3COOH \quad (15)$$
guanine

These four substances appear to be the only ones, amongst many tried, which these organisms can attack at a sufficient velocity to supply energy for growth and the plausible hypothesis was formed that the purines were oxidised by CO_2 with the formation of acetic acid. This was confirmed by the results of a fermentation carried out in the presence of radioactive C^*O_2. Radioactive C was recovered from the acetic acid (both in the methyl and carboxyl groups) and from the cell material, proving that acetic acid is formed by reduction of CO_2 and — taken in conjunction with other evidence — making it highly probable that all the acetic acid formed has this origin. Some radioactive carbon was also found in the cell substance showing that again the reduction of CO_2 provides material for cell synthesis (9).

In 1941 a novel form of fermentation by a thermophilic *Clostridium* isolated from horse manure was reported (12) in which 85 % of the glucose fermented appeared as acetic acid; the fermentation occurred in growing cultures and 5 % of the carbon of the glucose was calculated to be in the cells; no carbon dioxide was produced and no other product of fermentation could be demonstrated. It thus appeared that the fermentation conformed to the equation

$$C_6H_{12}O_6 = 3\ CH_3.COOH \quad (16)$$

The fact that the sole product of hexose breakdown was a 2-carbon compound and that CO_2 was absent made it impossible to account for this fermentation on the EMBDEN-MEYERHOF-PARNAS scheme unsupplemented by any other main reaction. BARKER (6) however associated the phenomenon with the WIERINGA reaction, suggesting that this fermentation is in fact an oxido-reduction in which the

CO_2 formed in the fermentation oxidises the intermediary products of hexose breakdown to acetic acid

$$C_6H_{12}O_6 + 2\ H_2O = 2\ CH_3.COOH + 2\ CO_2 + 8\ H \quad (17)$$

$$8\ H + 2\ CO_2 = CH_3.COOH + 2\ H_2O \quad (18)$$

Net reaction $C_6H_{12}O_6 = 3\ CH_3.COOH$ (19)

BARKER's hypothesis was confirmed by the use of heavy carbon C^{14} (8).

The bacteria were grown on the medium of FONTAINE *et al.* to which 0.2 % $Na_2C^{14}O_3$ was added; the vessel was sealed and incubated 3—6 days at 55° C. During the reaction 94 % of the C^{14} disappeared and 81 % was recovered in the acetic acid and in the cells. The C^{14} was distributed between the methyl and carboxyl groups of the acetic acid. This experiment leaves no doubt that the acetic acid arises by the reduction of CO_2. We have here in fact a reaction in which the hexose is completely oxidised the case being precisely analogous to combustion by molecular oxygen.

$$\begin{array}{l} \qquad\qquad\qquad\qquad 3\ O_2 \rightarrow 6\ H_2O \\ \qquad\qquad\qquad\qquad\quad \uparrow \\ C_6H_{12}O_6 \rightarrow 6\ CO_2 + 12\ H \\ \qquad\qquad\quad \downarrow \\ \qquad\qquad\qquad\quad 6\ CO_2 \rightarrow 3\ CH_3.COOH \end{array}$$

One might perhaps hazard the guess that we have here a fermenting organism endowed with the enzyme or enzymes necessary for the reduction of CO_2 to acetic acid. The CO_2 would then intervene as a hydrogen acceptor in the oxido-reduction steps of the fermentation cycle just as does molecular oxygen; triose phosphate then, instead of undergoing the usual form of oxido-reduction, would be oxidised by CO_2. If the relative rates of oxidation by CO_2 and of internal dismutation were such as to permit some accumulation of fermentation products at this stage these would become oxidised later. This possibility has already been demonstrated by BARKER (6) who showed that pyruvate is completely decomposed to acetic acid, the CO_2 arising from the pyruvic being in this case, in excess of that needed for complete oxidation, the whole reaction conforming to the equation

$$4\ CH_3.CO.COOH + 2\ H_2O \rightarrow 5\ CH_3.COOH + 2\ CO_2 \quad (20)$$

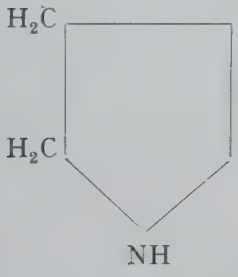
It now becomes obvious that any anaerobe possessing what one may term the „WIERINGA enzyme (or enzymes)" may be expected

so to oxidise its substrate that the whole of the mobilisable hydrogen appears as acetic acid. If the molecule attacked is more oxidised than carbohydrate CO_2 will also appear, if it is more reduced either some extraneous source of CO_2 will be used, or the oxidation will come to a standstill, or some product more reduced than carbohydrate may be expected to accumulate.

The reduction of amino-acids.

Non saccharolytic anaerobes are usually highly proteolytic and depend on the amino-acids produced in proteolysis to supply their energy for growth. In studying the metabolism of such organisms it is convenient to be able to distinguish which amino-acids function as hydrogen donators and which as acceptors. Using *Cl. sporogenes*, HOOGERHEIDE and KOCHALATY (19) used the hydrogen-hydrogenase reaction to detect the hydrogen acceptors and showed that six amino-acids were rapidly reduced by molecular hydrogen *viz.*, proline, hydroxyproline, glycine, ornithine, arginine and tryptophan.

With the exception of the first two — not properly speaking amino-acids — reduction was accompanied by deamination and the reaction proceeded rapidly. In the case of proline the ring is ruptured and the reduced product is δ-amino valeric acid; in the case of the amino-acids proper a reductive deamination takes place with the production of NH_3 and the corresponding fatty acid.



$$+ H_2 \rightarrow CH_2NH_2.(CH_2)_3.COOH \qquad (21)$$

$$CH_2NH_2.COOH + H_2 \rightarrow NH_3 + CH_3COOH \qquad (22)$$

With the exception of hydroxyproline and tryptophan those amino-acids reduced by hydrogen have been shown to function as hydrogen acceptors in oxido-reductions between pairs of amino-acids (STICKLAND reaction (35, 36, 40)).

Obviously if the products of the reduction of the amino-acid acting as acceptor have been established by the use of hydrogen the course of the oxido-reduction between a pair of amino-acids can be more readily disentangled; thus:

$$1 \text{ Alanine} + 2 \text{ proline} + 2\ H_2O \rightarrow 2\ \delta\text{-amino valeric acid} + 2 \text{ acetic acid} + NH_3 + CO_2 \quad (23)$$

$$2 \text{ Proline} + 4\ H \rightarrow 2\ \delta\text{-amino valeric acid} \quad (24)$$

By difference

$$1 \text{ Alanine} + H_2O \rightarrow 1 \text{ acetic acid} + CO_2 + NH_3 + 4\ H \quad (25)$$

Thus reaction 25 shows the expected products of the oxidation of alanine, which have in fact been verified (35).

Hydrogenlyases

Many bacteria are able, instead of effecting reductions by the use of molecular hydrogen, to effect oxidations by its liberation. Metabolically speaking these two reactions complement each other; the hydrogenase reaction permits an organism to reduce its substrate without using an organic hydrogen donator whilst the lyase reaction enables it to oxidise its substrate without a hydrogen acceptor.

The Key lyase reaction is of course

$$H.COOH \rightarrow H_2 + CO_2 \quad (26)$$

discovered by Pakes and Jollyman in 1901 (28) and shown by Woods to be reversible (39).

It was believed for a long period that formic acid was the only compound capable of this type of decomposition; it is now clear that the reaction is of wider distribution. It has, for example, been shown (42) that *Cl. tetanomorphum* can liberate hydrogen from glucose, glycerol, pyruvate, fumarate, and malate but is unable to attack formate in this way. Pyruvate was decomposed as in a typical butyric fermentation:

$$3\ CH_3.CO.COOH + H_2O \rightarrow CH_3.COOH + CH_3.CH_2.CH_2.COOH + H_2 + 3\ CO_2 \quad (27)$$

The same organism attacks the following amino-acids with liberation of hydrogen accompanied in each case by NH_3 and CO_2: glutamic acid, aspartic acid, serine, cysteine, histidine, methionine and tyrosine. The fact that NH_3 and CO_2 are liberated in each case suggests that this is the main method of attack on the amino-acid; moreover it was found that the amino-acids not attacked in this way were not decomposed at all by this organism, indicating that the hydrogenlyase form of decomposition is of fundamental importance in its metabolism.

Relationship between dehydrogenases and hydrogenlyases.

Discussion as to whether formic hydrogenlyase is a distinct enzyme or whether it is a combination of formic dehydrogenase and hydrogenase has hitherto turned on the relative distribution of these three enzymes amongst bacterial strains. The evidence is summarised by WARING and WERKMAN (38).

At best such evidence is indirect and inconclusive and can only carry conviction if sufficient cases are examined to place it on a statistical basis. Recently however something more concrete has been forthcoming, due to some observations of WARING and WERKMAN (38) who grew *A. indologenes* on a medium from which iron had been decreased by the use of 8-hydroxyquinoline to the low level of less than 0.003 μg/ml. A comparison of the relative activity of normal and iron-deficient cells with respect to hydrogenase, formic dehydrogenase and formic hydrogenlyase (total gas production) can be seen in Table I.

The activity of the enzymes in question appears to be abolished a fate shared by enzymes known to contain iron *viz.*, catalase, peroxidase, cytochrome and cytochrome oxidase. On the other hand other dehydrogenases (ethanol, lactic, malic, fumaric and succinic) were more active in the deficient than in the normal cells. The evidence is thus strongly in favour of iron entering into the composition of the enzymes in question.

A theory put forward by WARING and WERKMAN is as follows: „(1) Production of molecular hydrogen from formate by certain bacteria is accomplished by the combined action of (*a*) formic dehydrogenase; (*b*) an intermediate electron mediator; and (*c*) hydrogenase according to the reactions

$$HCOO^- + H_2O \rightarrow HCO_3^- + 2\,H^+ + 2\,e \qquad (28)$$

$$2\,H^+ + 2\,e \rightarrow H_2 \qquad (29)$$

(2) The electron carrier in question contains functional iron probably in a manner similar to cytochrome." This interesting hypothesis awaits further investigation.

It is obvious, however, that whatever may be the relation of formic dehydrogenase and formic hydrogenlyase a closely similar one must be expected between the dehydrogenases and lyases of glucose and amino-acids in *Cl. tetanomorphum* previously mentioned. It is also certain that the presence of iron in the growth medium conditions the formation of gas ($H_2 + CO_2$) producing enzymes.

Table I (cf. (38)).

Effect of iron deficiency on the enzymes of *A. indologenes* (grown anaerobically).

	$Q^{N_2}_{CO_2+H_2}$		
	Glucose	Pyruvate	Formate
Normal cells 1	306	108	248
,, ,, 2	434	110	162
Iron deficient cells 1	6	3	1
,, ,, ,, 2	9	3	2

	Q_{O_2}			
	Glucose	Lactate	Pyruvate	Formate
Normal cells	34	27	36	36
,, ,,	34	—	36	57
Iron deficient cells	49	15	13	10
,, ,, ,,	56	12	18	11

	Dehydrogenase Activity M.B. Decoloration time min.							
	Glucose	Formate	H_2	Ethanol	Lactate	Malate	Fumarate	Succinate
Normal cells	3	6	1.0	4.5	9.3	30	30	32
,, ,,	3.5	6.6	1.2	5.5	11	39	35	38
Iron deficient cells	8	> 180	> 180	11.2	16	71	74	104
,, ,, ,,	6	120	> 180	8.5	17	53	—	—

The importance of iron in this connection fits in with the observations of PAPPENHEIMER and SHASKAN (29) on the fermentation of *Cl. welchii*. The normal fermentation products of this organism are lactic, acetic, traces of butyric acid, ethanol, CO_2 and H_2; growth on a medium of low iron content decreases all the products except lactic acid which then forms the main fermentation product. It has also been shown with another strain of the same organism that fermentation by suspensions carried out in an atmosphere of C_O

result in complete inhibition of gas production, lactic acid then forming 70 % of the fermentation products (2).

The Rôle of Hydrogenase in Photosynthesis.

The *Athiorhodaceae* are those purple photosynthetic bacteria which reduce CO_2 anaerobically in the light by means of hydrogen transferred from organic compounds, generally fatty acids, the reaction being expressed by the equation (cf. 15, 16, 24):

$$CO_2 + 2\,HA = (CH_2O) + 2\,A \qquad (30)$$

The evidence that these organisms build up cell material solely from CO_2 reduced by organic hydrogen donators and not by any fermentative breakdown became overwhelming only when strains of these purple bacteria were found which were able to replace the organic hydrogen donators by molecular hydrogen (16).

$$2\,H_2 + CO_2 = (CH_2O) + H_2O \qquad (31)$$
$$-\triangle H = \pm 0 \text{ Cals.}$$

Members of the *Thiorhodaceae* are also known able to replace H_2S by H_2.

It has been shown conclusively by several workers that reaction 31 is anaerobic and dependant on light (13, 25), and the reaction has been used to determine the quanta of light energy absorbed in the reduction of 1 mol. CO_2. This turns out, rather surprisingly, to be the same as that required by the green plant where the overall reaction requires 114 Cals [1]); this provides a hint that it is not the transfer of 2 H to CO_2 either by hydrogen or any other donator which is the reaction utilising the light energy quanta. Additional information on this point is provided by the green algae of *Scenedesmus* and related genera. These organisms normally photosynthesise like other green plants (reaction 32); if held anaerobically in the presence of hydrogen for 1 hour their metabolism undergoes a change; this is evidenced by (1) the production of non volatile fermentation acids; (2) by the absorption of hydrogen (development or activation of hydrogenase); if nitrogen is the gas used hydrogen is evolved (appearance of hydrogenlyase). The short induction period suggests that both enzymes are present before the onset of anaerobic conditions and are brought into activity by an internal

[1]) $H_2 + CO_2 = (CH_2O) + O_2$; $\triangle H = 114$ Cals. (32)

reduction. This adaptation process in respect of the hydrogen enzyme is poisoned by cyanide (10^{-4} M) and by hydroxylamine (10^{-4} M); the production of fermentation acids is however unaffected by these poisons.

The adaptation process alters the type of photosynthetic mechanism used by the cell; on illuminating in the presence of H_2 and CO_2 both gases are now absorbed according to reaction 31 and no O_2 is liberated; no multiplication however occurs in these conditions. If now the intensity of illumination is increased reaction 31 slows down and is replaced by reaction 32, in other words the bacterial mode of photosynthesis (B) is replaced by the green plant mode (P). The two photosynthetic processes are clearly distinguishable by the action of poisons as shown in table II (cf. 17).

Table II.

Poison	Type of Photosynthesis	
	Bacterial (B)	Plant (P)
Cyanide (10^{-4} M)	Inhibits	Inhibits
Hydroxylamine (10^{-4} M) .	No effect	Inhibits
2, 4-dinitrophenol	Inhibits	Inhibits
Carbon monoxide	Inhibits	No effect

Thus hydroxylamine in low concentration appears to inhibit the liberation of oxygen which is part of the P mechanism but does not affect the reduction by hydrogen which is part of the B mechanism [1]); carbon monoxide on the contrary inhibits the reduction by hydrogen but has no effect either on the liberation or on the reduction of CO_2 in the P mechanism. These findings have contributed to the view that the photochemical action is similar in both cases and consists in the formation of a CO_2—H_2O complex which splits into a reduced product leading to carbohydrate and an oxidised product of a peroxide nature. So far the paths of both B and P processes are similar; the fate of the peroxide complex is different in the B and P cases; in the former it is reduced by hydrogen-hydrogenase (or other reducing system) in the latter it is decomposed by an enzyme system peculiar to green plants with the liberation of oxygen. To discuss this fully is outside the scope of

[1]) If it were shown that hydroxylamine were being reduced by hydrogen to NH_3 the significance of this inhibition would disappear.

this article and the competence of the writer but it is fully dealt with elsewhere (18, 26).

Apart from the light thrown on the photosynthetic mechanism the case of *Scenedesmus* is interesting from the standpoint of the evolution of the photosynthetic mechanism. May one hazard the suggestion that the bacterial form of photosynthesis is the more primitive and consisted in the development of a mechanism for using the solar energy to form a complex of CO_2 and H_2O of high energy content which gave rise to a reduced product leading to carbohydrate and an oxidised product which was reduced to water by hydrogen and hydrogenase. The green plant might then be supposed to have evolved by developing a second photosynthetic mechanism by which the oxidised half was split to water and oxygen instead of being reduced; the green plant thus relieved itself of dependence on organic compounds. This speculation of course implies that life started anaerobically in conditions where a variety of organic material formed part of its environment, an attractive supposition put forward ably by OPARIN (27); the first step towards aerobic life then would be the development of bacterial photosynthesis in shallow water at low light intensities, photosynthesis B and the second the mechanism for the liberation of oxygen at high light intensities by the development of photosynthetic mechanism P. This would lead to the development of plant life in regions where light intensely favoured the plant mechanism, and an environment of organic compounds was no longer necessary and would enable the plant to invade dry land.

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(Department of Microbiology, New Jersey Agricultural Experiment Station, Rutgers University, New Brunswick, N.J.).

CERTAIN ASPECTS OF THE PHYSIOLOGY OF ACTINOMYCETES [1])

by

SELMAN A. WAKSMAN

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Prologue

Since Beijerinck first attempted, nearly half a century ago to throw light upon the physiology of the actinomycetes and to establish their role in soil processes (3), the elucidation of the activities and importance of this large and widely distributed group of microorganisms was comparatively slow until very recent years, The lack of specific information concerning the intermediary metabolism and other physiological and biochemical reactions of the numerous species of actinomycetes becomes obvious, in the light of the corresponding advances made in our knowledge of the fungi and of the bacteria. It is true that considerable information accumulated during recent years on the morphology and systematic position of the actinomycetes, and their wide distribution in the soil and in other natural substrates. Despite the fact that hundreds of species have now been isolated and described and despite the ease with which most of these organisms can be cultivated on artificial media, their metabolism, however, has remained a rather obscure chapter in microbial physiology. Their role in natural processes has been a matter of speculation, and their practical utilization has hardly come into consideration.

Several reasons for this neglect may be suggested: The medical bacteriologist has had to deal with only a few disease-producing forms; these are relatively insignificant as compared to the bacterial and fungus diseases. The plant pathologist recognized only one major and one minor disease — potato scab and sweet potato pox; he, as well as the medical bacteriologist, were most concerned with

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the systematic position of these organisms, their type specificity, and practical methods of control; the organisms were grown on complex organic media and interest in their physiology did not go much beyond pigmentation characteristics. The soil bacteriologist and the general bacteriologist recognized the existence of a number of distinct morphological and physiological groups among these organisms and developed various synthetic media for the study of their cultural characteristics. They recognized that most actinomycetes are sensitive to an acid reaction, that they are able to produce various enzyme systems, that they comprise not only mesophilic but also thermophilic forms, and that they exert associative and antagonistic effects upon other groups of micro organisms. These investigators, as well, contributed comparatively little to the knowledge of the intermediary metabolism of the actinomycetes, and as to the possible importance and utilization of these organisms, they did not go much beyond BEIJERINCK.

BEIJERINCK pointed out, for example, that actinomycetes are omnivorous organisms, capable of living both under luxuriant conditions, from the point of view of nutrition, and in a very poor environment, even in distilled water in ordinary laboratory air. Water and air were said to supply nutrients for the modest needs of these organisms, although BEIJERINCK emphasized that actinomycetes are unable to fix atmospheric nitrogen. BEIJERINCK also demonstrated that actinomycetes produce traces of acid, probably lactic, in glucose media, and that they are able to reduce nitrate to nitrite. The last process was considered of doubtful importance in the soil; although it was believed that, under certain conditions, it may lead to losses of nitrogen through the interaction of nitrites with ammonium compounds. BEIJERINCK also emphasized the fact that the black pigment produced on protein media may function as an oxidizing agent; he tried to postulate, on this basis, the significance of actinomycetes in natural processes. He also recognized that actinomycetes occur in soil at considerable depths, where they may exceed in numbers the other groups of microorganisms; this he explained by their greater resistance to conditions unfavorable to their nutrition. He finally suggested that actinomycetes play an important role in the humification of the organic matter in the soil. BEIJERINCK thus laid the basis for all the subsequent developments in the study of the occurrence and activities of the actinomycetes.

Actinomycetes are able to utilize both inorganic and organic

forms of nitrogen; the extent of their growth upon artificial media is governed by the available energy, the supply of oxygen, available nitrogen, and certain other nutrient elements. One of the greatest contributions to a better knowledge of these organisms was their cultivation on synthetic media, upon wich they form characteristic morphological structures and upon which develop a variety of specific biochemical characteristics, such as pigments. Although their function in the physiology of actinomycetes is still open to question, these pigments have contributed much to the recognition of many specific types.

The present knowledge of the biochemical properties of actinomycetes, of their nutrition, and of other physiological characteristics, can be briefly summarized as follows: They are capable of readily breaking down proteins to amino acids and to ammonia; frequently, active proteolytic enzymes have been demonstrated. Many actinomycetes are strongly diastatic and some are capable of attacking cellulose, certain hemicelluloses and lignins, as well as fats and rubber-like materials. They are able to utilize a large variety of organic compounds for nutritive purposes and grow under various favorable and adverse conditions. Some are able to grow at temperatures as high as 60° to 65° C., especially in composts, wheras others, such as those prevailing in lake bottoms, thrive at rather low temperatures. Under unfavorable conditions they may grow only very slowly; this has often raised the question concerning their active participation in a given process. Most of them are sensitive to an acid reaction (pH 6.0) and are favored by an alkaline reaction (pH 7.0 to 7.5) of the medium. They produce oxidative and reducing enzyme systems.

With the rapid progress, within recent years, of our knowledge of antibiotic substances, and with the recognition that actinomycetes may play an important role in the production of such agents, new interest was aroused in the nutrition of these organisms. The introduction of the submerged culture method for their cultivation has made possible not only their rapid and abundant growth but also the study of many physiological reactions not previously recognized.

The problem

The present status of the physiology of the actinomycetes, their role in natural processes, and their practical utilization can be only briefly outlined here, with special emphasis upon certain phases

that still require elucidation. The great majority of actinomycetes are saprophytic organisms. Only very few forms are capable of infecting the animal body or of causing disease in plants. Their ability to cause damage to industrial products, such as textiles, and to produce undesirable effects in foodstuffs is very limited. The former consists, for the most part, in a partial weakening of the fibers; and the latter, in the formation of specific odors, which contribute to spoilage of such products as cacao.

Of the four genera now recognized among the actinomycetes — *Actinomyces*, *Nocardia*, *Streptomyces* and *Micromonospora* (13) — the animal pathogens are found largely in the first, namely the anaerobic genus, and to some extent in the second, and the plant pathogens are found in the third. The last three genera occur in great abundance in soils, where they make up nearly 25 per cent of the total population of microorganisms developing on the ordinary agar plate; in composts, especially at high temperatures; in drained peats; in lake bottoms; in the dust; and on the surface of grasses and foodstuffs. Their relative abundance in close proximity to the roots of plants is due not so much to their particular preference for living roots as to the fact they find nourishment in the dead residues and excreta of the roots, a fact already known to Beijerinck.

In order to obtain abundant growth, sufficient energy material must be supplied by proteins, carbohydrates, or organic acids; proper sources of nitrogen, either organic or inorganic, and certain minerals, notably potassium, magnesium, phosphorus, sulfur, and iron, are also necessary. The need for growth-promoting substances is still in doubt. For the production of specific metabolic products, as in the formation of streptomycin by *Streptomyces griseus*, the presence of organic precursors is essential. Certain forms are capable of producing vitamin-like substances favoring the growth o fother microorganisms.

Under comparable conditions of nutrition, actinomycetes may produce as much growth and decompose as much of the substrate as some of the common fungi and bacteria, as illustrated in Table I.

The effect of glucose upon the growth of the organism and upon the utilization of an amino acid as a source of energy and as a source of nitrogen is comparable to similar effects upon fungi (Table II).

The metabolic changes in the medium are greatly influenced by the nitrogen source (Table III).

These results bring out quite emphatically that actinomycetes

Table I.

Decomposition of different amino acids by microorganisms (15).

100 ml medium containing 1 per cent of amino acid

Amino acid	Organism	Growth, dry weight mg	NH_3-N produced mg
Glycine	*Streptomyces* sp.	59	31
,, ...	*Trichoderma* sp.	50	24
Alanine	*Streptomyces* sp.	126	39
,, ...	*Trichoderma* sp.	80	22
Glutamic acid . .	*Streptomyces* sp.	169	28
,, ,, .	*Ps. fluorescens*	128	29
,, ,, .	*Trichoderma* sp.	218	29

Table II.

Influence of glucose on decomposition of glycine by microorganisms (15).

1 % glycine in cultures

Organism	Glucose	Age of culture	Glycine-N decomposed	NH_3-N produced	Glucose decomposed	Growth, dry weight
	Per cent	days	mg	mg	mg	mg
Ps. fluorescens	2	5	26	3	820	99
Actinomyces sp.	2	8	36	19	570	213
Trichoderma sp.	2	5	49	1	1,490	804
Actinomyces sp.	0	8	4	6	—	23
Trichoderma sp.	0	6	6	8	—	30

Table III.

Metabolic changes and efficiency of carbon utilization of *S. lavendulae* in aerated cultures (18).

	Tryptone medium	Glycine medium
Mycelium, dry weight.	101 mg	106 mg
Glucose consumed	488 mg	782 mg
NH_3-N liberated	4 mg	22 mg
Nitrogen compounds deaminated . .	92 mg	162 mg
Lactic acid produced	126 mg	58 mg
Volatile acid as acetic	4 mg	13 mg
Conversion of glucose to lactic acid .	25.8 %	7.5 %
Conversion of glycine to acetic acid .		10.3 %
Efficiency of carbon utilization . . .	24.8 %	14.3 %

are similar to the bacteria and the fungi in their nutrition, in their energy utilization, in the transformation of nitrogenous compounds, in ammonia liberation, and in cell synthesis.

The ability of actinomycetes to decompose various carbohydrates and nitrogenous compounds (10, 11) suggests the capacity of these microorganisms also to attack complex plant and animal substances, as well as the more resistant humus materials. Amino acids, for example, are decomposed by actinomycetes as rapidly as by the fungi and the bacteria. Actinomycetes are also capable of attacking native proteins, both of plant and of animal origin (16). They are able to decompose cellulose, in the form of straw or other plant materials, as well as lignin (11). Because of this ability to attack resistant lignin, actinomycetes are apparently capable of decomposing resistant humus materials, as those found in soils and in peats (Table IV).

Table IV.

Decomposition of sedge and reed peat by microorganisms (11)

Seventy gm moist peat (20 gm dry) decomposing 28 days at 28° C.

Culture	CO_2 liberated mg C	NH_3-N mg	Nitrate N mg
Actinomyces sp. . .	87.7	11.6	1.8
Trichoderma sp. . .	88.4	13.3	0.8
Soil infusion	68.7	5.8	3.8

A pure culture of an actinomyces was found to be similar, in its ability to decompose peat, to one of the most active soil fungi, and each of them produced a greater effect than the complex soil population, as measured by the amount of CO_2 liberated and of ammonia produced. The decomposition of plant materials by actinomycetes is influenced by a number of factors, such as reaction, aeration, moisture content, temperature, and presence of other organisms.

Among the various biochemical activities of actinomycetes, the production of antibiotics or substances having antibacterial and antifungal properties has recently attracted considerable attention. Although it has been known since 1890 that certain actinomycetes have the capacity of interfering with the growth of other microorganisms, it is only in very recent years that any of the active substances involved have been isolated and that these substances have found practical application.

GASPERINI (5) was the first to demonstrate the antagonistic action of actinomycetes; however, he considered that the ability of an actinomycete to develop upon the surface of bacteria and fungi bringing about the digestion of their cell material, was largely of a parasitic nature. The fact that actinomycetes grow only slowly in natural soils frequently led to the suggestion that they constitute an important factor limiting bacterial development in the soil. LIESKE (8) demonstrated that actinomycetes are capable of antagonizing the growth of various bacteria; this action is selective in nature, affecting only certain bacteria, such as *Staphylococcus aureus*, and not others. GRATIA (6) must be credited with having made the first comprehensive study of actinomycetes as agents capable of lysing bacterial cells; however, his studies were carried out largely with dead bacteria, although living cells were later found to be affected also. These investigations led to the isolation of a concentrated preparation of the first antibiotic produced by this group of organisms, namely actinomycetin (17), a substance that proved to be a protein of comparatively little practical significance.

The first comprehensive survey of the distribution of antagonistic actinomycetes in nature was made by NAKHIMOVSKAIA (9). The results obtained emphasized the capacity of a large proportion of these organisms to suppress the growth of various bacteria. Of 80 cultures isolated from different soils, 47 possessed antagonistic properties, but only 27 were capable of producing antibacterial substances in the medium. The active agents — the antibiotics— thus produced inhibited the growth largely of gram-positive bacteria.

WAKSMAN *et al.* (14) came to the conclusion that antagonistic actinomycetes are widely distributed in nature, especially in soils and in composts. Of 244 cultures isolated by these investigators at random from different soils, 106 or 43.4 per cent were highly antagonistic. Similar results were obtained by examining a large series of well-identified organisms kept for a number of years in a type culture collection, as shown in Table V. The antagonistic organisms were largely represented by the genus *Streptomyces*.

As a result of these studies, there were isolated at the New Jersey Station 5 distinct antibiotics, namely, actinomycin in 1940, streptothricin in 1941, micromonosporin in 1942, streptomycin in 1943, and grisein [1]) in 1945. These substances vary greatly in their

[1]) Yet undescribed.

Table V.

Distribution of bacteriolytic properties among actinomycetes (17).

Preparation	Total strains	Activity against *S. aureus*			Growth-inhibition of *B. subtilis*		
		++	+	0	++	+	0
Number of strains	164	24	54	86	22	54	88
Per cent of strains	100	14.6	32.9	52.4	13.42	32.9	53.7
Number of filtrates	67	9	11	47	5	4	58
	Activity on heat-killed *E. coli* and *S. aureus*						
Number of filtrates	67	23	25	19	—	—	—

chemical nature, selective antibacterial properties, toxicity to animals, and chemotherapeutic potentialities (12). Some of the antibiotics are produced on simple synthetic or organic media; others require the presence in the medium of an organic precursor, although the organisms appear to be capable of synthesizing this precursor, if time is allowed. Of these antibiotics, streptomycin, because of its low toxicity and its activity against gram-negative and acid-fast bacteria, has found extensive application in the treatment of a variety of human diseases.

Actinomycetes also possess antagonistic activities against fungi (2). In a survey of the antifungal properties of 80 cultures, in which *Colletotrichum gloesporioides* was used as the test organism 17.5 per cent were found to be strong inhibitors, 38.8 per cent were weak inhibitors, and 43.7 per cent had no inhibiting action. There results are similar to those obtained in the above two surveys on the antibacterial properties of actinomycetes.

In addition to the aforementioned antibiotics, several others have been reported. It is sufficient to mention two. GARDNER (4) obtained as an air-contaminant a culture of *Nocardia* which produced an antagonistic effect against a variety of gram-positive bacteria; this antibiotic was designated as „proactinomycin". Another antibiotic designated as „mycetin" has recently been reported to have been isolated from *S. violaceus* by Russian investigators. Although compounds of several types have thus been isolated and described, the exceptionally large number of actinomycetes capable of producing antibiotics points to the existence of many other compounds still unknown.

Among the other problems bearing upon the physiology of

actinomycetes, the parasitic and saprophytic potentialities of these organisms have been investigated (1). In an effort to determine whether differences exist in the nutrition of forms capable of causing scab on potatoes, as compared to saprophytes, it was found that the parasites are able to utilize sucrose and raffinose, to be inhibited by ammonia, and to produce melanin pigments in a tyrosine medium, whereas many saprophytic forms do not show these reactions.

Epilogue

The actinomycetes represent a large and heterogeneous group of microorganisms with varied morphological and physiological properties. Their wide distribution in nature, their abundance in many substrates, notably in soils, peats and composts, the ability of some to cause certain plant and animal diseases, the capacity of many to decompose a great variety of organic compounds, and the property of a large number to produce antibiotic substances — fully justify a more comprehensive study of the physiology of these organisms. Recently, methods have been developed for growing actinomycetes rapidly and on a large scale, which will facilitate considerably such investigations. Actinomycetes offer a challenge to the geneticist who is interested in variations and mutations of organisms; to the physiologist who is interested in intermediary metabolism of microorganisms; and to the biochemist who is interested in unravelling the chemical reactions involved. The ease with which they can be cultivated and their ability to form certain interesting and possibly important compounds under controlled conditions of culture offer tempting invitations to a further investigation of this widely distributed and highly heterogeneous and most inviting group of microorganisms.

Literature.

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(Department of Agricultural Bacteriology, Macdonald College, McGill University, Que., Canada).

MICROBIAL ACTIVITIES IN PODSOL SOILS IN EASTERN CANADA

by

P. H. H. GRAY

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Microbiological studies have been made of several podsol soils of Eastern Canada. The greater area of the region known as the Eastern Townships is covered with these heavily leached soils. Those occupying areas over 400 feet above mean sea level are characterized by their high content of organic matter and low level of basic mineral constituents. They are well supplied with nitrogen, phosphorus, and sulphur. An analysis for a representative sample to a depth of 8" in the virgin state (1), will illustrate this (Table I).

Table I.

	Podsol	Heavy Clay
Loss on ignition . .	13.78 %	7.36 %
Organic carbon . .	6.62	2.62
Total nitrogen. .	0.30	0.16
P_2O_5	0.16	0.14
SO_3	0.13	0.10
MgO	0.65	2.58
K_2O	1.89	1.98
CaO	0.59	2.10
Lime requirement		
CaO lb per acre .	12700	2500
pH	4.46	6.14

Analysis of a representative sample of heavy clay soil, given in a parallel column, suggests that the outstanding difference between these two kinds of soil lay in the content and distribution of organic matter, and the percentages of calcium and magnesium. A description of the upper portion of the podsol soil profile follows:

Horizon
A_1 2″ black raw humus
A_2 3 to 6″ leached fine sandy loam
B_1 4 to 6″ red fine sandy loam.

The botanical cover is generally white birch and spruce, and in more open, less wooded, areas have developed massive hummocks of moss and lichen, interspersed with fern and „hand-hack" (*Spirea tomentosa*).

A large part of the area is under cultivation, but with relatively poor yields. The soils, after cultivation, are classified by the National Soil Survey Committee as Greensboro loam.

A microbiological analysis, by no means complete, has been made of the separate horizons of a few virgin soils.

The aim of these investigations was to ascertain if the ordinary bacteriological methods would be adequate to demonstrate a relationship between the organic matter and microbial activities.

For this purpose, samples were collected from visibly separate horizons with the least possible mixture of material of one horizon with the adjacent one. Physical and chemical analyses were made, in addition to the biological (2), (3). Physical and chemical characteristics of one soil are shown in Table II; bacteriological and other biological results from laboratory samples of the same soil are given in Table III.

The results suggest, in a manner which is brought out by no other type of soil, that the microorganisms are dependent upon the nutrient materials in the organic matter horizon of such virgin soils. The relative abundance of the microorganisms and their activity in the separate horizons may be compared with that in arbitrarily selected layers, of equivalent depth, in a virgin clay soil, in which the separate horizons could not be visually distinguished (Table IV).

The organic matter is more evenly distributed vertically in the clay soil.

An experiment was undertaken to ascertain if the material in the separate horizons were capable of supporting growth of microorganisms by supplying energy (carbonaceous material) or nitrogenous material, or both. Pressure extracts were prepared from soil of each horizon, and used as the nutrient base in an agar

Table II.

Physical and chemical characteristics of separate horizons of a Canadian podsol soil.

Horizon	Condition	Organic carbon %	Loss on ignition %	Water-holding capacity %	Hygroscopic moisture %	Ratio C : N
A_1	Organic matter	31.34	60.16	152.50	12.14	23.9
A_2	Leached (eluvial)	0.62	5.85	51.74	2.44	19.0
B	Illuvial	2.21	5.96	58.20	3.02	15.0

Table III.

Biological characteristics of separate horizons.

Horizon	Carbon dioxide mg per 100 g per 100 hr.	Bacteria per g	Actinomyces per g	Nitrate nitrogen ppm 29 days	NH_3 from urea 7 days
		in fresh samples (plate method)			
A_1	358.3	10,116,000	1,494,000	307.6	24.6
A_2	14.2	348,000	17,000	17.6	6.8
B	5.6	136,000	11,000	8.7	6.8

Table IV.

Biological activity in separate layers of a virgin clay soil.

Depth of sample, inches	Carbon dioxide mg per 100 g per 100 hr.	Bacteria per g
0—3	17.50	15,500,000
3—6	8.55	7,400,000
6—9	5.28	9,700,000

medium, alone and with added carbon as mannitol, or added nitrogen, as nitrate. A sample of soil was plated with THORNTON's medium (4) and with the agar prepared with the extracts. THORNTON's medium allowed the development of 87 colonies; the extract-media yielded colonies as shown below (Table V).

Table V.

Bacterial colonies in soil-horizon extract media.

Extract from horizon	Extract alone	Extract with mannitol	Extract with nitrate
A_1	99	106	112
A_2	78	65	81
B	70	62	68

The extract from the organic matter horizon yielded a few more colonies than did THORNTON's medium, but the difference was found to be statistically insignificant. Additional carbon and nitrogen both lowered the numbers in the media from the lower horizons when these are compared with the media from the upper horizon. It cannot be claimed, on this evidence, that the extracts were lacking in carbon or nitrogen. It has been long known, however, that the addition of readily available carbonaceous material to soils usually results in a great outburst of cell development, indicating that energy material is at a minimum in natural soils. The addition of nitrate does not encourage the development of new cells; soils appear to contain sufficient nitrogen in an easily available form, but a deficiency of assimilable carbon.

The experiments with these soils from which it was concluded that additional nitrate nitrogen had no effect on the activity of the microorganisms were part of a series, in three areas separated by several miles, in which individual components of the usual fertilizer mixtures were added to cultivated soils in the field (5). Neither bacterial numbers nor total activities were affected by nitrate, potash or phosphate, in each year during two years of treatment.

Since these soils have a high lime requirement value, the correction of this deficiency was relatively easy to do. Limestone, at six tons to the acre, improved the biological conditions, since the bacterial numbers, carbon dioxide, and nitrification of the soil

nitrogen were all stimulated after treatment with that amendment. The area in which the greater increases were observed yielded the following results in the second year after the lime had been added.

	Control plot	Limestone plot	Increase per cent
Bacteria	8.58	15.03	75.2
Carbon dioxide	22.03	49.03	122.6
Nitrates	30.25	43.09	42.4

From the standpoint of economy it was hoped, as one result of these early studies, to find some way of releasing the large amount of stored-up energy from the relatively intractable fraction of the organic matter. Limestone, however, though it had a great effect on the microbial complex, does not act as a solvent of soil organic matter. It was found in laboratory experiments (6) that certain strong alkali treatments increased the amount of soluble organic matter. The results of applying calcium oxide (an amount to satisfy lime requirement, or twice that amount), sodium carbonate, and sodium hydroxide to field soils were also examined. The total microbial activity was increased by all treatments, either singly or in combination. Calcium oxide was the most effective in stimulating the yield of carbon dioxide, the average yields from three or four samplings in each of two seasons being from 45 to 95 per cent higher than the yields from plots not receiving that chemical. Numbers of bacteria and the activity of the nitrifying bacteria were stimulated to a numerically greater extent. It would appear therefore that both assimilable forms of carbon and oxidizable nitrogen compounds are released from the soil organic matter by the solvent action of these alkali treatments.

The sufficiency of assimilable nitrogen in these soils was also shown in a series of laboratory experiments in which glucose was added, without and with additional nitrate (7). The ready availability of some part of the soil nitrogen was shown by the fact that 500 mg of glucose per 100 g of soil could be used up within 48 hours. The addition of 16.7 and 33.4 ppm of nitrate nitrogen did not increase the rate of utilization of glucose nor the amount of carbon dioxide developed in 12 days. The production of carbon dioxide was increased, however, in a sample in which during incubation the nitrate nitrogen had reached 40 ppm; the increase

was slight but possibly significant. It should not be assumed, however, that nitrate alone is a limiting factor, for during the process of nitrification other, at present unknown, ,,growth factors'' may be produced.

Studies have not yet been undertaken extensively on the nature of the micro-flora chiefly responsible for the decomposition of such easily assimilable organic materials in the soil itself, though a beginning has been made, following the work of Lochhead and his colleagues at Ottawa, upon the ,,nutritional groups'' of soil bacteria. A preliminary study on the nutritional requirements of one group, of twelve aerobic species of cellulose decomposing bacteria, isolated from Quebec soils, has shown how complex such a problem is, and how apparently impossible it is, at first analysis, to consider one such group as a homogeneous one.

The work reported briefly above was done mainly in collaboration or in close association with soil chemists; without such association and ,,fellow-workmanship'' the soil microbiologist cannot hope to derive much valuable knowledge of the conditions under which the microorganisms function in the breakdown of the soil organic matter.

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(From the Biochemical Institute, Helsinki).

COMPETITION OF *RHIZOBIUM* STRAINS IN NODULE-FORMATION

by

ARTTURI I. VIRTANEN and HILKKA LINKOLA

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1. Introduction.

A remarkable phenomenon which is observed when inoculation experiments are conducted with leguminous plants is the resistance caused by the nodules first produced by one strain of *Rhizobium* against later infections by other strains. We have made use of this phenomenon in order to obtain „control plants" for pot experiments in the greenhouse, *i.e.*, plants which do not receive atmospheric nitrogen through the activity of the root nodules. If an uninoculated pea is planted to a pot containing quartz sand and watered with N-free nutrient solution, effective nodules appear gradually on the roots — though not so rapidly as on the inoculated plants — and the „control plant" begins to grow. This is not prevented by sterilization of sand and seed *prior* to experiment because the danger of infection is particularly great in a greenhouse where experiments with legumes have been carried on for years. If, on the other hand, pea is inoculated with our ineffective strain H VIII, which is as rapid a nodule-producer as the effective strains, in most cases the pea does not grow at all. Only white round H VIII nodules are then found on its roots. In numerous carefully arranged experiments the said strain is proved to be completely ineffective (1).

The above phenomenon has already been treated earlier in literature. Hiltner (2) felt that a certain degree of immunity is set up by the entrance of the rhizobia into the plant and that only bacteria of a higher „virulence" than those already present can effect secondary invasion. Israilsky (3) came in his studies to the conclusion that the infection of the plant by one strain results in an immunity which inhibits infection by other strains. Miss Löhnis (4) observed that after a poorer strain had formed nodules

a more effective strain could still induce further nodulation. The reverse was not true. DUNHAM and BALDWIN (5) succeeded in securing simultaneous nodulation by effective and ineffective strains on the same plant. If a certain strain had already formed nodules on the roots, it was found much more difficult to induce nodulation by a different strain, although it sometimes succeeded. The degree of effectiveness did not effect the results. NICOL and THORNTON (6) disclaim altogether the immunizing effect of the first strain. In their careful experiments they have arrived at the result that the cause of the apparent immunity is simply ascribable to the fact that each strain is able to form a certain number of nodules in the root system. This number is formed rapidly by one strain and slowly by the other. After the roots have been „saturated" with nodules the same or a different strain is no longer able to form nodules on them. The experiments of NICOL and THORNTON were made with pea and soya bean. The plants were inoculated successively with two different strains. The first inoculation was made at the time of sowing, the second on pea 6 weeks after the first and on soya bean 9 weeks after the first inoculation

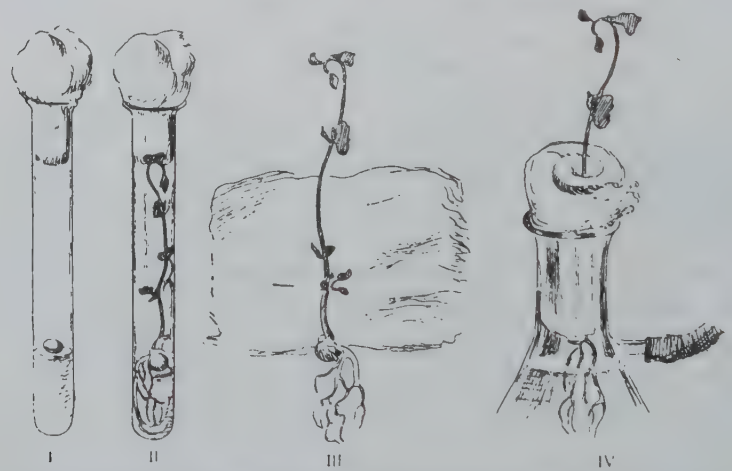

Fig. 1. Sterile culture system. Cultivation of sterile seedling (I—II) and transfer to culture flask (III—IV).

2. OWN EXPERIMENTS.

Without knowing the results obtained in Rothamsted we undertook during the war studies on the resistance caused in the roots by the first nodule-forming strain. In order to prevent with certainty all contaminations by foreign strains we performed these

experiments in the sterile culture system (Fig. 1) devised in our laboratory (7). By means of this the root system of the plant is guarded against infections by air, while the green parts are growing in the open air. Under these conditions the plants can grow excellently. In most favourable cases about 200 mg of nitrogen have been fixed per one inoculated pea.

As a substrate in our experiments we used partly quartz sand partly nutrient solution. In the latter case the formation of nodules could be visually followed. By gradual lowering of the level of the liquid in the culture flask a liberal supply of air was quaranteed to a large part of the nodules, and consequently, the red pigment, leghaemoglobin, appeared in most of the nodules formed by the effective strains shortly after nodulation. The nodules formed by ineffective H VIII were, however, always colourless, and could thus be easily distinguished from the effective nodules. Besides, the latter were elongated and larger in size than the H VIII

Fig. 2. A cluster of red, long nodules (marked with arrow) formed by effective H 7 strain on roots of pea where ineffective H VIII strain had previously produced round white nodules. H 7 nodules appeared only $1^1/_2$ months after inoculation when the plant had withered.

nodules (8). Fig. 2 illustrates the differences in the shape of the nodules.

H VIII forms a great number of nodules over a long period. The number of nodules also varies greatly in parallel water cultures with this as well as with some effective strains hitherto tested. Thus the observation of CHEN (9) that each strain forms a constant

number of nodules per gram of root could not be confirmed under these circumstances.

The technique employed in our experiments was the following. A sterile seedling of pea was inoculated with H VIII strain and transferred to a culture flask. The first nodules appeared usually within 8—12 days. In sand cultures the first nodule could not regularly be detected immediately when formed, but in water cultures it was easily observed. After the first nodules had been observed with certainty, the plant was inoculated again immediately or at least within a few days with an effective strain. In order to enable inoculation a glass tube closed with cotton wool had been passed before sterilization through the plug of cotton wool which closed the culture flask. Inoculation was made with a sterilized pipette through the tube, using as inoculum the water suspension of the particular strain of legume bacteria. In experiments with peas N-free nutrient solution of HILTNER was used (composition of the solution was: 10 l tap water + 2.5 g KCl + 2.5 g $Ca_3(PO_4)_2$ + 2.5 g $CaSO_4$ + 3.94 g $MgSO_4$ + 30 drops of 5 % $FeCl_3$ solution). The culture flasks were 1 l suction flasks, each containing about 2 kg quartz sand in experiments where sand was used for a substrate.

The pea variety used in our experiments was Torsdag pea of Svalöv. Seeds of approximately the same size were selected either by weighing or by measuring with the eye. According to our experiments the N-content of seeds, though of equal weight, varies considerably. For instance, seeds weighing 188 mg, whose N-content was determined according to KJELDAHL in five lots, 10 seeds in each, contained the following quantities of nitrogen expressed as mg per seed: 7.14, 6.99, 7.42, 6.71, 6.56, on the average 6.96, the N-content of seeds weighing 194 mg was respectively 7.21, 6.78, 7.49, 7.77, 7.21 mg, on the average 7.29 mg. Differences in the N-content of individual seeds were of course still noticeably greater. For instance, 10 seeds of Torsdag pea, each weighing 190 mg contained 6.86, 6.02, 7.14, 6.44, 6.72, 6.44, 7.14, 6.72, 6.86 and 6.86 mg nitrogen respectively (on the average 6.72 mg) and 10 seeds each weighing 210 mg contained 7.28, 7.56, 8.26, 6.86, 7.56, 7.42, 7.98, 8.12, 7.98 and 8.12 mg (on the average 7.71 mg). As in most of our experiments the weight of seeds varied from 200 to 220 mg, variations of 2 3 milligrams may occur in the N-contents of individual test plants though no N-fixation has occurred.

The experiment 1 shows that the strain H 11 did not induce nodulation in 17 days when it was applied to pea after the first

Table I.

Experiment 1 (28.IX—30.X.42). Substrate: quartz sand. Seeds selected by measuring with the eye. Plants transferred to culture flasks 28.IX.42. Artificial and natural light.

No. of plant	Inoculation	N-nutrition $Ca(NO_3)_2$	Dry weight of plants	N in plants		Observations
		mg N	g	mg	%	
1	Not inoculated	—	0.908	6.8	0.75	No nodules
2	,, ,,	—	0.560	8.1	1.44	
3	,, ,,	—	0.455	6.9	1.53	
4	H VIII	—	0.625	9.2	1.47	Round white nodules
5	H VIII	—	0.600	8.0	1.33	
6	H VIII	20(19.X)	0.853	20.8	2.44	
7	H VIII (29.IX) + H 11 (13.X)	—	0.526	7.4	1.40	Only white H VIII nodules, no H 11 nodules
8	H VIII (29.IX) + H 11 (13.X)	—	0.530	7.5	1.40	
9	H 11	—	1.465	33.3	2.27	Red elongated nodules
10	H 11	—	1.502	29.6	1.97	

H VIII nodules had appeared. No. 6, to which 11 days before the end of the experiment 20 mg nitrogen were added in the form of $Ca(NO_3)_2$, shows that still at this stage the plants were able to grow and to utilize nitrogen. This preliminary experiment was made in collaboration with Leena Kahra and O. Aaltonen.

In experiment 2 the effective H 11 strain was not able to form nodules during a month after the appearance of the first H VIII nodules. H VIII continued to form nodules over the whole test period which indicates that „saturation" of roots with nodules of this strain could not be the cause of non-appearance of H 11 nodules. If the root system was inoculated at the start simultaneously with H VIII and H 11 (plants no. 12—14) the majority of nodules on two of the plants was formed by H VIII and on one by H 11 and the N-fixation was respective. Thus the competition between two strains gave different results in parallel replicates.

The results of experiment 3 are in accordance with the previous experiment. Uninoculated plant no. 2 was inoculated with H 11 at the same time as the plants which already had H VIII nodules. Nodulation on no. 2 was noted for certain after 12 days, which shows that the plant still became infected as easily as in the seedling stage. The non-ability of H 11 to form nodules on plants, which

Table II.

Experiment 2 (25.I—15.III.43). Substrate: quartz sand. Weight of seeds 200—220 mg selected by weighing. Sterilized seeds were placed in agar tubes 15.I.43, plants transferred to culture flasks 25.I.43. Artificial and natural light. Plants in full bloom 15.III when harvested.

No. of plant	Inoculation	Dry weight of plants	N in plants		Observations
		g	mg	%	
1	Not inoculated	1.017	9.2	0.9	No nodules
2	,, ,,	1.045	10.8	1.0	
3	H VIII (25.I)	0.654	9.0	1.4	White, round nodules
4	H VIII (25.I)	0.768	10.0	1.6	
5	H VIII (25.I)	0.971	11.1	1.1	
6	H VIII (25.I) + H 11 (12.2)	0.869	10.2	1.2	Only white H VIII-nodules. H 11 did not form nodules
7	H VIII (25.I) + H 11 (12.2)	0.742	10.3	1.2	
8	H VIII (25.I) + H 11 (12.2)	0.732	9.2	1.2	
9	H 11 (25.I).	3.025	97.4	3.2	Red oblong nodules
10	H 11 (25.I)	1.840	55.0	3.0	
11	H 11 (25.I)	2.306	73.3	3.1	
12	H VIII + H 11(25.I)	0.785	13.2	1.7	Chiefly white nodules of H VIII, a few red H 11 nodules
13	H VIII + H 11(25.I)	0.895	15.1	1.7	
14	H VIII + H 11(25.I)	3.970	100.1	2.5	Red H 11 nodules dominant

already had H VIII nodules, is therefore not due to the ageing of plants.

Experiment 4 (21.VII—7.IX.43) which was similar to the above one gave corresponding results, thus it is unnecessary to record it here.

In experiment 5 where a nutrient solution was used for substrate, nodule formation could be followed daily. The peas were harvested when in pods. By that time the uninoculated control plant and all the inoculated plants which were not supplied with nitrogen had completely withered. In accordance with the previous experiments it was observed that in a fortnight none of the effective strains used formed nodules on peas which had first been inoculated with H VIII, though a distinct nodulation occurs within the same time

Table III.

Experiment 3 (31.III—26.V.43). Substrate: quartz sand. Weight of seeds 200—220 mg, selected by weighing. Sterilized seeds were placed in test tubes 24.III and the plants transferred to culture flasks 31.III.

No. of plant	Inoculation	Dry weight of plants	N in plants		Observations
		g	mg	%	
1	Not inoculated	0.800	11.1	1.4	No nodules
2	H 11 (15.IV)	1.353	42.8	3.2	Red oblong nodules visible 27.IV
3	H VIII (31.III)	0.732	9.2	1.2	
4	H VIII (31.III)	0.721	11.0	1.5	
5	H VIII (31.III)	0.924	9.2	1.0	White round nodules
6	H VIII (31.III)	0.697	12.7	1.8	detected 10-15.IV
7	H VIII (31.III)	0.924	11.2	1.2	
8	H VIII (31.III)	0.874	10.6	1.2	
9	H VIII (31.III) + H 11 (15.IV)	0.831	12.9	1.5	Nodules detected 10-15.IV, only white
10	H VIII (31.III) + H 11 (15.IV)	0.888	13.5	1.6	H VIII nodules, H 11 did not from
11	H VIII (31.III) + H 11 (15.IV)	0.577	10.0	1.7	nodules
12	H 11 (31.III)	2.463	82.9	3.4	Nodules detected
13	H 11 (31.III)	1.646	52.9	2.2	9-15.IV
14	H 11 (31.III)	2.793	94.3	3.4	Red elongated nodules

on uninoculated peas of the corresponding age. H 2 formed no nodules at all on plants possessing already H VIII nodules though the experiment lasted over $2\frac{1}{2}$ months, whereas H 7 in one case formed some nodules on 2 months' old peas and the withered plant pushed out a new shoot which began to grow. Infection with strain H 6 caused nodulation on two peas a month after the appearance of the first H VIII nodules. On two peas again H 6 formed nodules $1\frac{1}{2}$ months after inoculation, when a new shoot appeared on the withered pea.

H VIII strain forms new nodules in water cultures at least within a month after the appearance of the first nodules, which indicates that the roots do not become „saturated" in a week following the inoculation during which time the effective bacteria should have entered the roots and the nodule formation should

Table IV.

Experiment 5 (1.XII.44—19.II.45). Substrate: nutrient solution. Seeds of equal size were selected by measuring with the eye. Sterilized seeds were placed in test tubes 23.XI.44 and the plants transferred to culture flasks 1.XII.44. All the plants, excluding the control (No. 1) were inoculated with strain H VIII. The first nodules were visible in different flasks on 8—11.XII. After this second inoculation was made with strains H 2, H 6 and H 7 on dates indicated in table. Plants were harvested 19.II.45 when pods had developed.

No. of plant	Inoculation	Dry weight of plants	N in plants		Observations
		g	mg	%	
1	Not inoculated	0.539	7.8	1.4	No nodules
2	H VIII (1.XII) + H 2 (9.XII)	0.529	8.5	1.6	Only white H VIII nodules
3	H VIII (1.XII) + H 2 (9.XII)	0.664	9.5	1.4	
4	H VIII (1.XII) + H 2 (9.XII)	0.864	12.2	1.4	
5	H VIII (1.XII) + H 6 (8.XII)	0.624	12.8	2.0	Red H 6 odules at the end of January
6	H VIII (1.XII) + H 6 (8.XII)	1.683	34.6	2.8	
7	H VIII (1.XII) + H 6 (8.XII)	1.106	32.8	3.0	Red H 6 nodules, detected 8.I
8	H VIII (1.XII) + H 6 (8.XII)	2.371	50.6	2.1	
9	H VIII (1.XII) + H 7 (13.XII)	0.630	8.6	1.4	Only white H VIII nodules
10	H VIII (1.XII) + H 7 (13.XII)	0.486	7.8	1.6	
11	H VIII (1.XII) + H 7 (13.XII)	1.496	37.8	2.5	Red H 7 nodules at the end of January

occur at full rate. It must be noted that a week after the appearance of the first H VIII nodules they are only few in number and the majority of the nodules is formed in the course of the following weeks. Moreover, it must be mentioned that whenever new nodules are formed by effective strains on ageing pea they occur scattered in the centre of the root system (*cf.* Fig. 2).

In order to find out what happens if the roots of pea are divided

into two culture flasks and the roots in the one are inoculated with the ineffective strain H VIII and, after the appearance of nodules in this branch, the other is inoculated with an effective strain we have carried out experiments in the so-called branched tube system developed by us. As this technique offers many possibilities for elucidation of nutrient uptake by plants and as a detailed description of it has not been given previously we shall give it here.

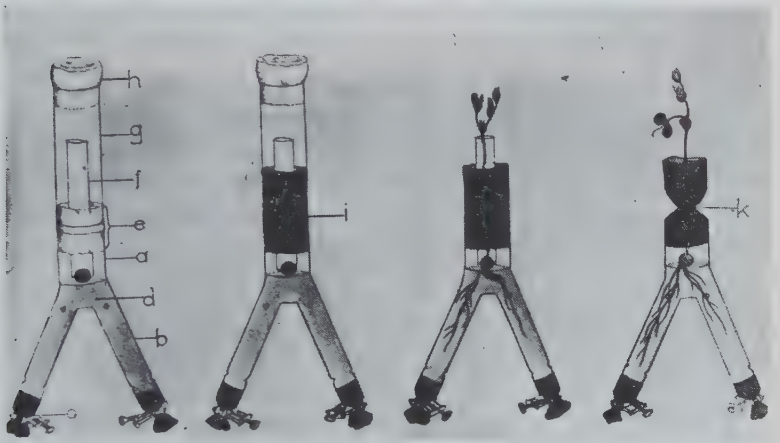

Fig. 3. Different phases of sterile branched-tube system. Detailed description in text.

The upper part (*a*) of the branched glass tube (Fig. 3) is 5—6 cm in length and 3 cm in diameter, the branches (*b*) 8 cm in length and 1.5 cm in diameter. The measures may vary according to the size of the plant to be grown. The lower ends of the branches are closed with a piece of rubber tubing and pinchcock (*c*). The tube is filled with 1.5 % agar (*d*) up to the level shown in the figure. After this the upper end of the tube is plugged with cotton wool (*e*) through which a glass tube (*f*) of about 1.5 cm in diameter and 9 cm in length is passed so that its lower end comes to a distance of about 2 mm from the surface of the agar. A 3 cm wide and 11 cm long glass tube (*g*) is fitted above the branched tube so that the cotton wool closes also the lower end of this tube. The upper end of the tube is also plugged with cotton wool (*h*). Over the joint of the tubes a piece of rubber tubing (*i*) is pulled. The whole apparatus is autoclaved at 120° C. The seed of pea is sterilized with sublimate, washed with sterilized water and after removal of the cotton wool plug (*h*), by means of sterilized forceps, is placed through the narrow inner tube (*f*) on the surface of the agar. When the seed germinates the plant grows up through the narrow inner tube and the roots grow into the agar dividing later into the branches of the tube. When the plant reaches the cotton wool it

is transferred to a nutrient solution. The upper glass tube (*g*) is removed as well as the narrow tube (*f*) whereby the green parts of the plants are freed. The wide rubber tubing is tightened with a string (*k*) around the lower cotton wool plug, in order to press the cotton wool tightly against the plant and to prevent the entrance of bacteria into the roots. The branches of the tube are dipped in alcohol for sterilization, and the pieces of rubber tubing are rapidly removed. Two 1 l suction flasks containing nutrient solution have been sterilized at 120° C. The branches of the tube are passed through the sterilized cotton wool which closes the flasks. The roots may now grow freely in their nutrient solutions under sterile conditions.

In the course of the experiment the agar may shrink a bit and comes out of the tube. For preventing this the branches of the tube are provided with small notches. The roots are protected against the harmful effects of light by means of black crape paper which covers the whole system from the rubber tubing downwards.

The branched tube system is rather inconvenient especially therefore that a part of the experiments must always be discarded. The dividing of roots into tube branches is incidental. If, again, the roots grow into the one branch only, the experiment is of course worthless. On the other hand, the system offers new possibilities for elucidation of nutrient uptake by plants and is applicable to the solving of many different problems.

One difficulty has been met in examining nodule formation in the branched tube system. It has been shown that bacteria may wander through the agar from one culture flask to the other, though the agar in the tube is unbroken. This does not occur regularly. Especially in winter when the temperature does not rise over 20° C., the other branch of the roots remains often nodule-free over the whole growing period, whereas numerous nodules are formed in the other branch after inoculation. In summer, on the other hand, the multiplication of bacteria on the surface between the roots and agar and their rapid wandering from one flask to the other is almost a regular occurrence. Partly for this reason the experimental data are somewhat contradictory. New experiments are therefore still needed.

In this connection it is out of place to record in full the results of the 9 experimental series which we have performed up to the present. We shall only briefly state the main points.

1. In some experiments the formation of the ineffective H VIII nodules on the roots in one culture flask prevented nodule formation by the effective H 2 strain on the roots in the other flask,

when the former branch was inoculated right at the start with H VIII and the latter only after the appearance of the H VIII nodules on the former branch. The immunity is in this case indisputable.

2. In some experiments the effective H 2 strain was able to form red active nodules on the roots in one flask when applied immediately after the appearance of the first H VIII nodules on the roots in the other flask. H 2 nodules appeared frequently within 8—12 days after inoculation. No immunity could be noted in this case.

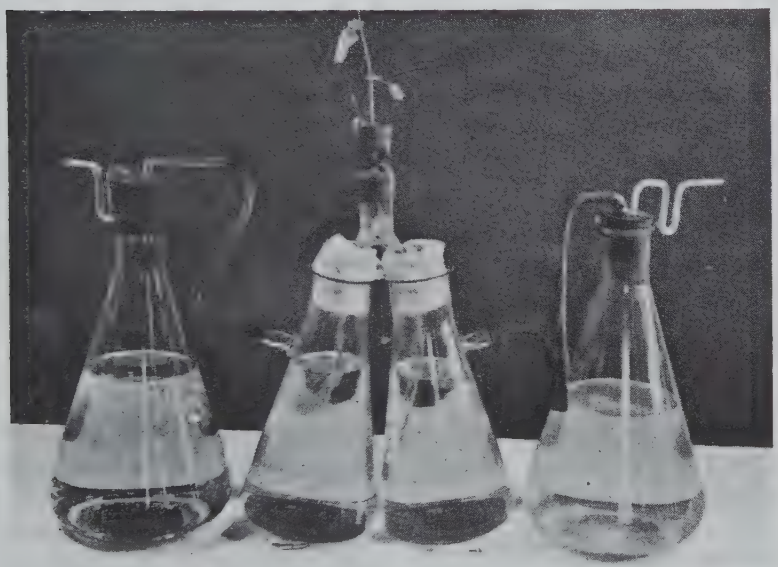

Fig. 4. Final phase of sterile branched-tube system. Roots of the plant divided into two culture flasks.

3. Conclusions.

The experiments described in the foregoing show that nodule formation by the ineffective strain H VIII has prevented nodulation by effective strains within the period during which nodules usually appear on uninoculated plants. Secondary inoculation usually does not lead to nodulation, though in some cases a few nodules may occur on ageing plants. Different bacterial strains show variations in this respect. The results are not interpretable by assuming the first strain to „saturate" the roots with nodules, thereby preventing later nodulation, since H VIII itself was found

to form new nodules in the course of many weeks. Neither can it be expected that H VIII would destroy the new strains in the substrate outside the root system, since nodules formed by these strains may later occur on ageing peas. The simplest and most natural explanation is that the strain first entering the roots immunizes the plant against other strains. During the ageing of plant immunity seems to decrease.

Another explanation is offered by the assumption that the effective strain used for later inoculation has truly formed nodules, but being white and ineffective these cannot be distinguished from the H VIII nodules. No evidences, though, are afforded to this conception.

The branched tube system described in this paper was used for solving the problem whether a secondary inoculation with an effective strain induces nodulation in that part of the roots which has not previously been infected by ineffective strain. The results of different experiments were contradictory. In certain experiments, though, nodule-formation occurred normally which never was the case when the whole root system was first inoculated with an ineffective strain.

Summary.

By the use of a sterile culture system experiments have been carried out with peas in order to elucidate to what extent inoculation with an ineffective bacterial strain effects the ability of an effective strain to produce further nodulation. It has been established that effective strains, when applied to the plant after the formation of the first nodules by an ineffective strain, altogether fail to form nodules or the nodulation is delayed very much. Differences are noted in this respect between different bacterial strains. The results can be explained easiest by assuming that the strain first entering the roots causes an immunity in the plant against later infections. Other possibilities for explanation are also discussed. The conception that the resistance would be due to the „saturation" of roots with nodules formed by the first strain which would inhibit further nodulation is not in accordance with the results recorded.

A method has been introduced for cultivation of plants under sterile conditions by dividing the roots into two or more culture flasks. This method is of great value when the nutrient uptake of

plants is elucidated. Successive inoculation with ineffective and effective strains gave varying results by this technique. The experiments seem to imply that if only a part of the root system is inoculated with an ineffective strain, immunity does not regularly occur against later infections by effective strains. This suggests that the immunity is a somewhat local phenomenon.

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(Department of Chemistry, New York University, New York).

SOLID SACCHARASE PREPARATIONS

by

CARL NEUBERG and IRENE S. ROBERTS

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In a just published monograph on the enzyme saccharase (1) we have referred the knowledge brought to light by investigators in all parts of the world during 113 years of research into the occurrence, preparation, properties, various types, utilization and behavior of this enzyme. Until to-day it has not been possible to isolate pure saccharase, much less crystallized one. Instructions how to make saccharase preparations are numerous; partly they are complicated, partly they require much time.

In what follows we wish to describe 2 methods for the preparation of solid invertase, which is both suitable for practical purposes, *i.e.*, for utilization as specific analytical reagent and also for industrial requirements, and is extremely stable.

A.

It has been known for a long time that the saccharase of yeast cells can to a large extent be brought into solution by autolysis in particular by the method of accelerated plasmolysis with subsequent autolysis introduced by HUDSON and associates (2). For a long time too processes have been in use, which first bind the dissolved and sometimes dialysed enzyme on to a suitable adsorbent and then redetatch it from the still damp adsorbent by means of elution.

The enzyme in its adsorbed form converted into the dry state does not seem to have been used in practice. Up to now saccharase has generally been available in the liquid form, *e.g.*, in 50—60 % glycerin solution. The use of solid invertase adsorbates may be recommended; their preparation is simple.

The well known adsorbents, such as charcoal, ferric hydroxide, aluminium hydroxide C, arsenic sulfide, zinc sulfide, strontium hydroxide, lead phosphate, bentonite, must be excluded as carriers for solid and stable invertase

preparations. Some of them are poisonous, some form undesirable and mean-looking by products; on some the adsorbed enzyme does not keep when stored.

As we found, adsorbates of invertase on calcium phosphate, and in general on water insoluble alkaline earth salts of acids of the group of citric, tartaric, saccharic and mucic acid, are free from these drawbacks.

The use of adsorbed invertase is possible since the enzyme is just as effective in the bound state as in the free form. HEDIN (3), MICHAELIS and coworkers (4), ERIKSSON (5), MEYERHOF (6), GRIFFIN and NELSON (7), WILLSTÄTTER (8) and others have shown that the state of dispersion of the enzyme is without influence on the activity. Indeed, this has only been proved for wet material, but we have ascertained that it applies to dried invertase preparations as well.

Example 1.

5 kg pressed yeast of the top or bottom fermenting type and as dry as possible are placed in 10 liters of tap water at room temperature. 250 ml chloroform and 250 ml toluol or 250 ml ethyl acetate and 250 ml toluol are added together with 100 grams of solid, finely powdered calcium carbonate. Instead of the chloroform etc. any other organic or inorganic substance with plasmolyzing effect can be used. The mixture is liquefied by agitating or shaking it. Baker's yeasts are allowed to stand for 5 days, for brewer's yeasts only 3 days are required. At the beginning the cover should be lifted from time to time. At last the mixture is centrifuged or filtered by suction. According to the type of yeast used the resulting liquid will be light or dark yellow. It is preserved under toluol, so that it can be kept without difficulty.

After adjusting the pH to 7 with NaOH a fresh solution of 27 gram ferric chloride ($FeCl_3.6\ H_2O$) in 1 liter water is added in one portion to each 600 ml of the above liquid. No attention need be paid to a slight precipitate, which sometimes forms. Immediately following the addition of the ferric salt a solution of 7.8 gram primary sodium phosphate ($NaH_2PO_4.2\ H_2O$) and 18 grams secondary sodium phosphate ($Na_2HPO_4.12\ H_2O$) in 1 liter water is added [1]). Since top fermenting yeasts are purer $^1/_3$ to $^1/_2$ of the reagents mostly suffices. A flaky gelatinous precipitate of ferric phosphate now forms and is removed by filtration or centrifugation.

The resulting clear solution of invertase (solution *a*) is odorless and only slightly yellow. It is carefully neutralized by dropwise addition of 25 % ammonia as long as Merck's universal indicator

[1]) Instead of the sodium salts equivalent amounts of potassium or ammonium compounds may be used and instead of the iron salt aluminium chloride.

remains yellow. It must not be allowed to turn into a greenish shade. The ferric phosphate now precipitated removes coloring matters and impurities from the solution and after filtering an almost colorless invertase solution is obtained (solution *b*).

The two precipitates are combined and suspended in a solution of 70 grams primary sodium phosphate ($NaH_2PO_4.2\ H_2O$) in 1 liter water. Shaking or agitating for about one hour dissolves most of the invertase adsorbed by the precipitates. The filtered liquid is carefully neutralized with 25 % ammonia. If a few flakes form they are filtered off; a third clear invertase solution is thus obtained (solution *c*).

The main solution (*b*) is now almost neutralized with 25 % ammonia and this is followed immediately by addition of a solution of 100 gram calcium chloride ($CaCl_2.6\ H_2O$) in 2 liters water. Agitating constantly this solution is then precipitated with the invertase solution (*c*). Any slight precipitate which might have formed on addition of the calcium chloride is disregarded. The precipitate of calcium phosphate obtained on addition of solution *c* contains the main quantity of invertase and is centrifuged or filtered off. After washing with H_2O in the centrifuge till free from chloride it is dried as fast as possible, *e.g.*, on clay or with acetone, and immediately and intimately mixed with 10 grams of dry acid potassium phosphate (KH_2PO_4). A small amount of the solid potassium phosphate is poured on top of the mixture to be on the safe side. The mixture, which now contains secondary calcium phosphate is dried completely in a dessiccator. The acid potassium phosphate may be replaced by other stabilizers, such as potassium bitartrate or saccharate, tartaric acid, mucic acid, citric acid etc. If a sample of the precipitated material is suspended in distilled water and tested with Merck's universal indicator the color should be yellow without any greenish shade. If at all possible all the precipitations and treatments with ferric chloride, phosphate, calcium chloride, ammonia etc. as well as the mixing with the mono potassium phosphate and the drying should be completed the same day.

After drying the material is finely powdered. It will retain the same activity for many months. 600 ml of invertase solution yield 50—60 grams of dry substance. If 100 ml of an 8 % saccharose solution are treated at pH 4.5 with 0.2—0.5 gram of the dry product at 37° C. inversion up to 0 rotation, *i.e.*, splitting of cane sugar to 75.75 % is completed in about 10 minutes or less.

Any other soluble alkaline earth or aluminium salt can be substituted for calcium chloride and iron. Strontium or magnesium can replace calcium. Instead of phosphoric acid other non poisonous acids which form insoluble alkaline earth salts, *e.g.*, citric or tartaric acid can be used. The method of preparation corresponds exactly to the one outlined above.

If the invertase adsorbed by the first two precipitates is not to be collected, the invertase solution *b* is treated directly with

100 grams of di sodium phosphate dissolved in about 2 liters of water. The remaining procedure is as before.

Example 2.

A simpler and shorter process can be used if light colored yeast is available.

1000 ml of the autolyzed plasmolysate are mixed with 20—30 ml m $CaCl_2$ and with 40—60 ml m/2 Na_2HPO_4. The precipitate settles rapidly after shaking. It is filtered off and the enzyme precipitated from the clear filtrate with 200 ml m $CaCl_2$ and 400 ml m/2 Na_2HPO_4. The precipitate can be filtered or centrifuged off easily and the subsequent procedure is exactly as described before.

Example 3.

1000 ml of autolyzed plasmolysate are mixed with 14 ml m $AlCl_3$, then with 14 ml m $(NH_4)_2HPO_4$. A water clear filtrate is obtained after removal of the aluminium phosphate precipitate. This is treated with 210 ml m $SrCl_2$ and 420 ml m/2 K_2HPO_4 as described above.

Example 4.

1000 ml plasmolysate are evaporated in vacuo to 200 ml. The temperature must not exceed 35° C. A precipitate is filtered off, if necessary, then 200 ml of m K-Na-tartrate solution (Rochelle salt) is added as well as 50 ml of a 4 m $CaCl_2$ solution. At rest the precipitate increases in quantity and it can easily be filtered off or centrifuged. It contains the invertase and is dried and stabilized as outlined.

B.

A simple solution of the problem ought to be possible, if, instead of binding invertase on added carriers one could fix it, without any loss, on the natural constituents of the cells and convert such a material into a dry and stable product.

A method of preparing acetone dried bottom yeast has been described by Albert, Buchner and Rapp (9) in 1902. It is based on treating brewer's yeast with acetone and subsequent drying. With the resulting preparation, known as „Zymin" Buchner obtained saccharose fermentation, so that it must have contained some invertase. However, prepared in this way, acetone dried yeast does not retain its efficacy when stored. Alcohol-ether dried yeast prepared in an analogous manner (10) equally loses its efficacy, losses which include invertase.

It has been known for a long time that invertase is very sensitive against alcohols (11) and various authors (12) have reported that

fact. WILLSTÄTTER in his collected papers especially stresses the destruction of invertase by acetone and alcohol. U.S. Patent No. 1.990.505 treats the use of various alcohols in preparing yeast containing invertase.

We now found that certain ethers able to dehydrate pressed yeast are suitable for the preparation of completely stable invertase products.

Such ethers are 1,3 dioxane (m-dioxane), 1,4 dioxane (p-dioxane), dioxolane, dimethyldioxane. Since the 2 last mentioned ethers are not sufficiently soluble in water they must be mixed with small amounts of a glycol, *e.g.*, ethyleneglycol, propyleneglycol, trimethyleneglycol, propanetriol etc.

Dioxane can also be mixed with ordinary ethyl ether and the mixture is suitable for the preparation, provided that the ratio is so adjusted that addition of approximately 10 % water does not cause separation into layers. 2—3 parts of dioxane to 1 part of ethyl ether would be a suitable proportion. After the invertase preparation has been filtered off by suction or filter press or centrifuged off the dehydrating solvents used may be recovered very easily.

The invertase preparation is dried over calcium chloride and paraffine, preferably under high vacuum. The preparation so obtained has the full inverting power of the yeast used as starting material and since it does not contain more than 2.3 % water, while well pressed yeast contains about 70—73 % water it is more active than the same quantity of the raw material. Apparently the product can be kept indefinitely. It has been found equally effective after a period of $2\frac{1}{2}$ years.

The use of completely neutral ethers eliminates the harmful action of the hydroxyl groups of alcohols and of the carbonyl groups of ketones. The yeast which has been dehydrated with dioxane can be washed with dry ethyl ether. This treatment may be recommended since drying can be carried through faster after removal of the adhering dioxane. The zymatic system is generally no longer effective, probably because coenzymes and activators have been dissolved out.

Instead of dioxane the methyl or ethyl ether of ethylene glycol (methyl resp. ethyl cellosolve) may also be used. Although these ethers contain a hydroxyl group they do not damage the enzyme, provided they are pure and adhering remnants are washed out of the invertase preparation with ethyl ether. The dry material pre-

pared with methyl cellosolve from fresh yeast sometimes still shows power to ferment.

While the chief effect of acetone or alcohol-ether is that of dehydration, dioxane and ethylene glycol monomethyl ether also cause plasmolysis. Pressed yeast is liquefied in a short time by stirring it together with $^1/_5$ resp. $^1/_{10}$ of the above mentioned substances. If such plasmolysates are poured into 5 times their own volume of the respective liquids a powdery, easily filtered enzyme material is obtained.

The method of treating certain raw materials with dioxane etc. can be applied to all microorganisms, plant and animal cells containing invertase, *e.g.*, the various species of *Aspergillus*, *Fusarium*, *Monilia*, *Mucor*, *Penicillium*, *Termobacterium mobile*, *Torula*, leaves of sugar beets etc.

The monoalkylethers of ethylene glycol and glycerine combine the properties of glycols and of ethers. Since they are neutral liquids, miscible with both water and several organic solvents they are well suited for dehydrating both enzymes and intact cells. They may also be used for preserving enzyme solutions. Much the same applies to dioxane. Until application was made for a patent (13) dealing with this subject, dioxane has never been recommended for the preparation of invertase. In a book by SUMNER and SOMERS (14) published soon afterwards, dioxane is mentioned as a substance which may be useful when crystallizing partly purified enzymes.

Example 1.

1 kg top or bottom yeast of approximately 27—30 % dry content and in as small pieces as possible is added, at room temperature with constant stirring and in small portions directly to 6 liter anhydrous dioxane. This is stirred for 10 minutes and then filtered by suction or centrifuged off. The residue is treated once more with 2 liter dioxane, stirred for 5—10 minutes and filtered off in whatever manner is convenient. The solid residue is washed, preferably right on the Büchner funnel or in the centrifuge, with 1 liter anhydrous dioxane and dried under vacuum at room temperature over calcium chloride and paraffine. To hasten the drying operation the product may be washed with anhydrous ether after the last treatment with dioxane. The yield is about 300 grams of a white porous mass or powder which can be finely pulverized without difficulty. If stored dry the invertase so prepared will keep for years.

Example 2.

10 gram of *Aspergillus oryzae* are centrifuged, washed, partially dried in the dessiccator on clay, pulverized and gradually added

to 60 ml of anhydrous dioxane. The material is stirred for 15 minutes, then centrifuged and treated once more for 10 minutes with dioxane, stirring continuously and finally centrifuging. The residue is washed with 20 ml anhydrous dioxane directly in the centrifuge. Drying is accomplished in the same manner as described above for the preparation obtained from yeast (example 1). Yield: 4 gram.

According to both methods saccharase preparations are obtained which are colorless (when using brewer's yeast as starting material sometimes with a tinge of yellow), odorless and tasteless. They are therefore suitable directly for any industrial utilization of the enzyme. Furthermore the dry preparations may be applied analytically, for the determination of sucrose in products of the sugar industry. The requisite quantity is so minute, that the determination of sugars, whether polarimetric or by reduction, is not affected by the presence of foreign substances. Blank experiments are superfluous unless it is a question of determining the very smallest amounts of saccharides. In both cases enzymes accompanying invertase in yeast remain admixed. In preparations made from bottom yeast according to method B the melibiase needed for the complete hydrolysis of raffinose is not lacking.

The essence of all previous results (1) is that the invertase is the less stable, the farther purification has been carried. Intentionally foregoing purification the two processes described above have been worked out. They yield useful products.

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(Soil Microbiology Department, Rothamsted Experimental Station, Harpenden, England)

THE BIOLOGICAL INTERACTIONS OF *RHIZOBIUM* TO ITS HOST LEGUME

by

H. G. THORNTON

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The practical importance to agriculture of the legume nodule organism needs no emphasis. Legume crops have been included in systems of agriculture from the earliest times and in all parts of the world. Their importance is due both to their high protein content and to their ability to grow in nitrogen deficient soils and to benefit other crop plants grown in rotation or in association with them. They owe their virtues to the symbiotic association of the nitrogen fixing bacteria of the genus *Rhizobium* in their root nodules.

„Species" and Strains of *Rhizobium*.

In their general morphology and growth characters the nodule bacteria resemble *Achromobacter radiobacter* and *Phytomonas tumefaciens* while in some characters they also resemble the Alkaligenes group (Conn, Wolfe and Ford (11)).

Attempts to classify strains of *Rhizobium* into specific groups have given rise to a considerable literature (for references see Fred, Baldwin and McCoy (15)). These attempts have been influenced by an early discovery of great practical importance — that of legume host specificity. Bacteria isolated from the nodules of a given legume species will infect certain other legume species or genera but not others. This fact is the reason for the need to supply a suitable strain of *Rhizobium* to a legume crop that is sown in a soil deficient in nodule bacteria appropriate to it. It is indeed chiefly in areas into which a new legume crop has recently been introduced that this practice of „inoculation" has met with its main success. As a result of laboratory experiments in cross inoculation, some 20 host specific groups of nodule bacteria have so

far been described. These groups vary considerably in their validity. The more truly specific groups have been elevated into the rank of „species" but even amongst these, cross infection has occasionally been observed (Wilson (46), also Kleczkowska, Nutman and Bond (24)).

It is also possible to classify strains of *Rhizobium* into different groupings by using other criteria. The results of cross agglutination tests between strains show that, for example, the clover nodule bacteria fall into several quite different antigen groups which will not cross agglutinate (Hughes and Vincent (16)), but that some strains of bacteria from clover nodules are antigenically related to some pea nodule bacteria, which form a separate cross inoculation „species" (Kleczkowski and Thornton (25)). There is a similar lack of correlation between host-infection groups and groupings based on susceptibility to specific bacteriophages (Kleczkowska 21, 23)) and on nutritional behaviour *in vitro* (Jensen (18)). Thus the prevalent „specific" grouping of the nodule bacteria according to their host legumes, while of practical convenience, is not necessarily natural in the sense of having any phylogenetic implication.

The strains of *Rhizobium* that can be isolated from a single host-plant species, or sometimes from a single individual plant, may not only differ in serological and cultural characters and in their susceptibility to bacteriophage but may also show large differences in their ability to benefit the host by nitrogen fixation, an important character which is discussed below.

The Organism in the Soil.

Rhizobium not only inhabits the legume plant but normally lives as a soil inhabitant, and can live for a number of years in field soil without the presence of its host plant. Both in culture and in the soil the organism passes through a series of changes in cell morphology which includes uniformily staining rods, banded rods and coccoid forms, the latter develop flagella and have sometimes been named „swarmers". In this motile stage the bacteria can migrate through soil at the rate of about 2 cm in 24 hours (Thornton and Gangulee (40)). It is no doubt in this stage that the organism reaches the roots of the host plant. It has been found that calcium dihydrogen phosphate stimulates the formation of the swarmer stage and that its presence also increases the number of nodules formed.

Itano and Matsuura (17) found evidence that the bacteria are chemotactically attracted to the roots.

The multiplication of *Rhizobium* in the neighbourhood of the host plant root is also stimulated by secretions from the root system (Nicol and Thornton (29)). The nature of these root secretions is not known, but there is evidence in the case of lucerne (*M. sativa*) that they are first produced by the seedling plant, at the time when the first true leaf develops, at which time the first nodules normally appear (Thornton (36)). The rate of multiplication of the bacteria in the root surroundings under standard conditions differs according to the strain. Where two strains are there present, acute competition takes place between them, which may result in the suppression of one of the strains, normally that having the slower growth rate. In such cases selective competition outside the root may determine which strain shall predominate in the nodules subsequently formed (Nicol and Thornton (29)).

Infection of the Legume Root.

It is well known that infection by *Rhizobium* normally takes place through the root-hairs and that these are deformed before infection. This deformation commonly takes the form of a curling of the root-hair tip produced by a small clump of the bacteria formed on the outside of the hair, close to its extremity. Deformation of the root-hairs can also be produced by a sterile filtrate of a suspension of *Rhizobium*. In this case it is less localised and produces a wavy or even branched growth of the root-hairs and a considerable increase in their length (Thornton (39)). The deforming action of the bacterial secretions is not specific to the group of legume plants that a given bacterial strain can normally infect (McCoy (26)). There is evidence suggesting that the active principle in the bacterial secretions may be indolyl-acetic acid (Chen (7)).

The actual infection of the root-hair normally occurs in the region of the deformed tip, the bacteria after penetration growing down the inside of the hair as a thread of slime containing organisms in the rod stage. There is always a strong limitation of infection, only a small percentage of deformed hairs actually becoming infected (McCoy (26)). A further limitation affects the subsequent development of the infection, but few of the infected root-hairs actually resulting in nodules.

The number of nodules produced under standard conditions is

a characteristic of the bacterial strain and differs greatly between strains (Chen (8)). Where two bacterial strains are simultaneously supplied to the root this differing infectivity may determine the proportion of nodules produced by each strain if these two strains do not compete selectively outside the root system (Nicol and Thornton (29)). A result of this limitation of infection is that where two strains are successively applied to a root system, the first applied strain may saturate the nodule forming capacity, after which no more nodules will be produced either by the same or by the second strain (Nicol and Thornton (29)).

The number of nodules formed by a given bacterial strain is also influenced by characters in the host-plants which are genetically controlled (Nutman (31)). Other inherited characters in the host-plant, fortunately rare, may even render it completely immune to infection by bacteria normally able to infect that plant species. The mechanism producing this limitation or complete inhibition of infection is not known. If a strain of *Rhizobium* is applied to a legume species of a cross inoculation group that it cannot infect, the root-hairs are deformed, but no bacteria can be formed inside the root-hairs. In this case therefore inhibition takes place at the cell wall of the deformed root-hair.

The infection is also greatly influenced by conditions of the environment. Thus the supply of nitrates to the root-system may check or inhibit infection and nodule formation. This is associated with the fact that nitrates check the deformation of the root-hairs whether by living bacteria or by their secretions (Thornton (39)). The ability of the secretions to deform the hairs is restored if sugar is supplied together with nitrate so that the important condition for root-hair deformation and infection would seem to be the ratio of available carbon to available nitrogen in the root-hair. Thus the extent to which nitrate application will reduce nodule formation in the field may depend on growth conditions that affect photosynthesis.

Formation of the Nodule.

The thread of slime containing the bacteria, usually called the „infection thread", after passing down the root-hair and reaching the root cortex, ramifies through the parenchyma cells. It sometimes penetrates the endodermis entering the cells of the root pericycle. The near presence of the infection thread causes the cells of the root to become meristematic and induces rapid cell-

division. This is presumably due to the secretion of a stimulant to cell division which may again be indolyl-acetic acid (THIMAN (34, 35)). WIPF (50) and WIPF and COOPER (51, 52) have observed that the dividing cells in young nodules have tetraploid chromosome numbers. They found such tetraploid cells scattered through the cells of the root cortex even in uninfected roots and their observations suggest that the formation of a nodule may be conditioned by the chance occurrence of tetraploid cells in the cortex near the point of penetration of the infection thread. Dividing cells of the root cortex can sometimes be seen to be entered by the infection thread but soon after infection the host cell ceases to divide and increases in size.

It is not certain how the infection thread penetrates the host cell-walls, but apertures in the cellulose walls can be shown to exist, through which it may gain entry. The infection thread is at first naked but soon it becomes encased in a sheath in the regions inside the host cells, while remaining without a sheath between the cells. Microchemical tests show that this sheath has a similar chemical composition as the host cell wall so that it is almost certainly deposited by the host cell (McCOY (26)). Bacteria are either released from the infection thread into the cytoplasm of the host-cell before its encasement or afterwards by swelling and bursting of the sheath (THORNTON (37)).

This process of host-cell infection by means of infection threads is normal in most legumes but in certain plants such as lupins an interesting variation occurs in which the bacteria are released into dividing cells and distributed amongst these by the process of mitotic division of the host cell (MILOVIDOV (27, 28)).

After their release the bacteria multiply to fill the cytoplasm and subsequently they increase in size and usually assume branched or pear-like shapes, in which state they have been described as „bacteroids". The shape of these bacteroids is characteristic of the individual bacterial strain (CHEN and THORNTON (10)). The young nodule is at first a mass of dividing cells but later it becomes differentiated into zones, an outer zone or more usually a distal cap of sterile dividing cells, an underlying zone in which the cells are entered by the bacteria and a central mass of tissue in which the cytoplasm of the infected cells is filled with the „bacteroids". This last region, sometimes called the „bacterial tissue", contains the bulk of the bacteria in a developed nodule and is doubtless the seat of nitrogen fixation.

This zonation of the nodule is accompanied by the out-growth of vascular strands each surrounded by its sheath of endodermis, from this central cylinder of the root, with the result that the nodule is placed in vascular connection with the rest of the host plant.

Activity of the Nodule.

The nitrogen that is fixed in nodules reaches the bacteria by diffusion from the soil atmosphere. The chemical process by which the nitrogen fixation is brought about is still not certainly understood in spite of an extensive literature on the subject. The solution of this problem seems most likely to be reached by investigations such as have been commenced at Wisconsin using an identifiable nitrogen isotope (Burris and Miller (16); Burris, Eppling, Wahlin and Wilson (5)). A critical discussion of the various theories as to the nitrogen fixation process is outside the scope of this essay nor is this the right time for such a discussion, whose conclusions would probably be invalidated in the near future by the results of work using „tagged" nitrogen. The position in 1940 is excellently described by Wilson (47). One is tempted to regard the discovery of haemoglobin in active nodules (Keilin and Wang (20)) as providing a key to the understanding of nodule physiology. Virtanen (43) has observed that under conditions unfavourable to nitrogen fixation the haemoglobin is converted into a green pigment. It may be that the function of the haemoglobin is to stabilise the oxidation-reduction potential in the nodule. This view was held by Peitz (32) who however mistook the chemical nature of the pigment. It seems also that traces of molybdenum are helpful to nitrogen fixation (Bertrand (2)). Indeed in Australia, there are molybdenum deficient soils where the addition of this element stimulates leguminous crops (Anderson (1)).

It has been shown by Virtanen and his collaborators that a considerable amount of combined nitrogen may be excreted from nodule bearing roots in the form mainly of aspartic acid (Virtanen (42)). He has developed a theory of nitrogen fixation based on these observations according to which the aspartic acid is derived from an oxime formed by the combination of hydroxylamine with oxalacetic acid. Virtanen and Laine (43) identified this oxime amongst the products of secretion. Other workers have obtained conflicting results as to the secretion of nitrogen compounds by legume roots, but their work corroborates that at Helsinki in

showing that such a secretion can take place under certain conditions, although it does not always occur (for references see Wilson (47)). These conditions are not yet fully specified but seem to be connected with the light supply to the host plant (Wilson and Wyss (48), Bond and Boyes (3)). This subject has an importance to the practice of mixed cropping since the secreted nitrogen can be taken up by other plants growing with the legume. The preferences of various plants for specific amino acids, recently studied by Virtanen and Linkola (45), has an obvious bearing on this practice.

Disintegration of the nodule centre.

After an active life of very variable duration, the central tissue of the nodule undergoes disintegration. This most interesting process is the result of the bacteria becoming actively parasitic upon the nodule. It commences normally near the base of the nodule, at the points where the original infection threads pass from cell to cell (Thornton (38)). The bacteria grow out from these points into the middle lamella of the cell wall which swells and becomes filled with bacteria. Soon after this the cytoplasm of the host cell is destroyed and the contained bacteria become lysed. Finally breakdown of the cell wall structure causes complete disintegration of the bacterial tissue. This process extends distally so that finally the centre of the nodule is completely destroyed and may even become hollow. Such disintegration is the normal fate of old nodules but it can be induced prematurely in young nodules by changes in the plant's physiology. Thus in *Vicia* growth in boron-deficient solution causes the development of nodules that are deficient in vascular strands and in which the bacterial tissue disintegrates very early (Brenchley and Thornton (4)). The same effect can be produced by keeping the plant in the dark (Thornton (38)). Since both these methods result in cutting off the supply of energy material to the nodule it seems reasonable to suppose that in both cases the change to parasitism is due to the withholding of the normal food supply to the bacteria.

Bacteriophage.

It is claimed by Demelon and Dunez (14) that a lysis of the bacteria in nodules can be brought about by bacteriophage and indeed that this is an important cause of the failure of stands of lucerne.

A number of workers have studied *Rhizobium* bacteriophage (see Vandecavaye and Katznelson (41), Katznelson (19), and Kleczkowska (21, 23)), which is indeed a convenient subject for investigation owing to its slow action which enables the stages of growth and attack to be followed easily.

Some of these bacteriophages are specific to a small number of strains of the host *Rhizobium* a fact that much reduces their importance in a natural soil that contains many strains of *Rhizobium* capable of infecting the legume crop, but may assume an importance where only one such strain, introduced by inoculation, is present in the soil. Demelon and Dunez (14), on the other hand have found relatively non-specific bacteriophages especially from lucerne *Rhizobium*, and claim that they harmfully affect the development and activity of the nodules. Nodule bacteria, however, very readily develop phage-resistant mutant forms and will do this not only in culture media but also in the soil (Kleczkowska (22)).

The high specificity of some bacteriophages makes them useful as a means of identifying individual strains of *Rhizobium*.

Ineffective Strains of *Rhizobium*.

If a number of strains isolated from the same legume species are compared, they are found to vary greatly in their ability to benefit the host plant by nitrogen-fixation, a fact first clearly shown by Stevens (33) and by Wright (52). In the case of some legumes such as clover and peas, strains can even be found that confer no visible benefit on the host-plant in a nitrogen deficient medium. When these ineffective strains were discovered it was thought that their ineffectiveness was due to some failure in the biochemical mechanism of nitrogen fixation, but this was hard to demonstrate since even strains effective in the host plant will not fix significant amounts of nitrogen in artificial culture.

Chen and Thornton (10) however followed the course of development in nodules produced by effective and ineffective strains of bacteria on clover, peas and soy beans and found that, in their material, the differences in total nitrogen fixed could be accounted for by the relative volume and duration of the active bacterial tissue. In ineffective nodules the total volume of infected cells was far smaller than in effective nodules. Even more striking was the difference in duration of this active tissue. In effective clover nodules disintegration of the nodule centre ultimately occurred as described above, but the process did not commence until these

were a month old and was not complete until after at least 2 months. In clover nodules produced by an ineffective strain complete disintegration of the bacterial tissue occurred after 10 to 14 days. When allowance was made for these two factors of volume and duration, it was found that the quantity of nitrogen fixed by a unit volume of active infected cells in unit time was the same for ineffective as for effective nodules. Thus the large differences in total nitrogen fixed could be wholly accounted for by failure of the ineffective strain to develop sufficiently or to last long enough in the tissues of the host plant. There is some evidence that this is in turn due to the formation, induced by ineffective nodules, of substances toxic to bacterial growth (Chen, Nicol and Thornton (9)).

The effectiveness of nodules produced by certain strains of *Rhizobium* would thus seem to be mainly if not wholly accounted for by a lack to symbiotic adaptation between the bacterium and the host plant, liable to be disturbed by either partner. As mentioned above, abnormal host plant growth induced by boron deficiency or by etiolation can cause early disintegration and consequently ineffective operation of the nodules. Nutman (30) has found that clover plants may possess characters controlled by recessive genes that will produce a completely ineffective response with a normally effective bacterial strain. These genes are highly specific towards individual strains of *Rhizobium*, producing an ineffective response with certain strains but not with others. Some clover plants possess hereditary characters that improve the activity of bacterial strains of low effectivity although very ineffective bacterial strains have remained ineffective on all clover plants on which they have been tested. In view of the existence of genes in the plant affecting nodule activity, it is not surprising that a number of workers have found that different species and varieties of plants respond differently to a single strain of *Rhizobium* that can infect them all.

Bacterial variants causing changes in effectiveness.

The nodule bacteria have a tendency to produce variant forms differing from their parents in effectiveness in the host plant. The tendency for an effective strain to produce ineffective variants is greatly increased by cultivation in certain soils. Thus Nutman (31) using an effective strain that had been passed 12 times through the clover host plant without any change in effectiveness found that storage for nine months in sterilised sandy soil caused the

appearance of a highly ineffective variant that comprised 30 per cent of the population. The type of soil greatly affects this tendency for ineffective variants to appear; although the soil factors concerned have not yet been specified.

It seems to be more difficult to induce the appearance of bacterial variants showing an effectiveness greater than that of their parent strain. NUTMAN however found that, on infecting clover with the ineffective variant produced by soil culture, two nodules out of 22,000 examined grew to a large size and contained effective reversions.

THE PRACTICAL PROBLEM OF INEFFECTIVE STRAINS.

Ineffective strains are abundant in certain leguminous plants in some districts. A geographical survey of strains of clover *Rhizobium* over Great Britain has shown that ineffective strains produce about 30 per cent of the nodules on white clover growing sparsely in hill districts of Wales, the North of England and Scotland, where they may thus be an important factor limiting the establishment of clover. Their abundance thus suggests the possibility of improving the clover content of hill pastures by inoculation with „effective" strains. The use of inoculation for this purpose, however, at present meets with two difficulties.

The first in the tendency, mentioned above, for effective strains introduced into certain soils, to produce ineffective variants. When the soil conditions that encourage the appearance of these variants can be specified it is possible that this difficulty may be met by soil treatment. Or stable strains not so liable to produce variants, may be discovered.

The second difficulty arises from the fact that inoculation in this case aims at replacing a natural population of ineffective strains by an effective strain necessarily introduced in much smaller numbers. The introduced strain must therefore face that competition in the soil with other strains, that has already been discussed. Fortunately strains vary greatly in their ability to compete with other strains, so that selection of a strain for seed inoculation that grows rapidly in soil may meet this second difficulty.

Preliminary field experiments with clover seed inoculation have in fact shown that a strongly growing strain can establish itself in the nodules in competition with the „wild" nodule bacteria in the soil while other strains fail to do so. This introduces a new factor

that should control the selection of a strain of *Rhizobium* for use in seed inoculation. It should not only be effective in fixing nitrogen, and genetically stable in this respect, but it should also have the ability to withstand competition from other strains of *Rhizobium* in the soil. Professor DEMELON would add to these requirements a resistance to the less specific bacteriophages.

Conclusion.

The intensive study of the symbiosis of *Rhizobium* and its legume host-plant has emphasised how delicate is the equilibrium upon which a satisfactory association depends. The effectiveness of the bacterial strain for a particular host plant, the genetic stability of this strain and its ability to compete with other strains in the soil may all influence the growth of the crop. The plant begins to exert its influence on the bacteria by root secretions before they infect the roots and both the degree of infection and the activity of the nodules that are formed, will be influenced by genes in the plant as well as by environmental conditions that affect its physiology. It is the separation of these complex factors, that is at once the main difficulty and the chief fascination in the study of this symbiosis.

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(Institut for Teknisk Kemi, Copenhagen).

ON THE ENSILING OF LUCERNE BY MEANS OF LACTIC ACID FERMENTATION

by

S. ORLA-JENSEN, ANNA D. ORLA-JENSEN and AGNETE KJAER

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When the first named author from the periodical „Antonie van Leeuwenhoek" received the honourable invitation to contribute to the festive publication for Professor KLUYVER, my collaborators and I had not finished any new work. However, as I am very eager to celebrate my illustrious colleague at the Technical University of Holland with a contribution from the Technical University of Denmark, I feel obliged to break my principle never to publish anything not completely concluded. Our ensiling method has, however, been so thoroughly checked in the laboratory that further studies of this problem will hardly change the results. Only a more detailed identification of some of the bacteria found is still outstanding and will be published later.

By ensiling it was originally understood to pack something into a silo. The expression has, however, gradually changed its meaning and it is now applied to acidification of green forage, independent of whether this is done in a silo or in an earth-pit only: The oldest form of silage known in Denmark is probably that of the diffusion slices from the sugar manufactories, which were kept in earth-pits. Since these slices have been heated, it certainly would have been appropriate to add lactic acid bacteria. On the other hand, when raw plant material is used we may expect that abundant lactic acid bacteria generally are present on the surface of the plant pieces. In the case of sauerkraut, for example, which is the earliest form of silage known, we thus failed to improve the product by inoculation with the lactic acid bacteria typical of this speciality. From ancient times has been proceeded quite correctly: the cabbage was first cut into small pieces: subsequently, some salt was added in order to make the juice and the sugar extravasate the cells and, finally, the whole mass was loaded with planks and stones. By this procedure, lactic acid fermentation sets in rapidly; the plant slices do not come in touch with the air and, thus, also the development

of aerobic micro-organisms, especially moulds, which oxidize the acid, is avoided.

Acidification is a natural and harmless means of preservation not only for plant-, but still to a greater extent for animal products. Sour milk is almost unlimitedly durable if it is protected against air and, consequently, against moulds. It is due to acidification that the fresh cheese mass suffers a favourable ripening process instead of a spoiling process of putrification. If only a pH 5.3 is reached in the beginning, we are on safe ground. Even meat assumes this pH soon after slaughtering, however, this is not due to lactic acid bacteria but to enzymes present in the meat. Owing to the low sugar content of meat, the pH increases rapidly. Provided the weather is not too hot, this short-lasting acidification makes it nevertheless possible to keep the meat without spoiling until it is tender. In this connection, we cannot abstain from mentioning that acidification has a preserving effect upon our food not only before it is consumed, but also after its consumption, by preventing the contents of the large intestine from putrefaction.

Actually, the ensiling of greater crops of fodder is not the consequence of theoretical considerations, but simply of the fact that certain fodder substances contain so much water that they cannot be dried by means of the usual methods of haymaking. This applies especially to the green maize in North America where, from ancient times, the preparation was made very rationally by cutting the plants into small pieces and tramping them into high cylindrical silos. Moreover, in many European countries, ensiling was frequently used for pieces of beet and beet leaves, grass (especially second crops), leguminous plants, etc. From the end of the last century, a vast literature is available regarding both the silage problem and the bacteriology of silage.

It was soon discovered that if milk is used for cheese manufacturing the cows should not be fed on silage, because through contamination with dung the milk becomes so rich in butyric acid bacteria that the cheese, especially Emmental cheese, will blow up. Originally, a distinction between sour and sweet silage was made. These terms, however, have nothing to do with the pH, but exclusively with the odour, sour smelling silages besides lactic acid also containing appreciable amounts of volatile acids, chiefly butyric acid.

Just as in a compost-heap, an increase in temperature occurs in the silage; generally, however, the temperature does not rise over 30—40° C. At the start, the increase in temperature is preponderantly due to the respiration of the living plants while, when a temperature of circa 45° C. is reached, it must be ascribed to the effect of thermophilic bacteria. These bacteria frequently have an optimum at 60° C. and cannot endure more than 70° C. Since both respiration and the thermophilic bacteria need oxygen, it is possible in large silos to regulate the temperature by means of pressure, *i.e.* the higher the pressure, the lesser the increase in

temperature. The water content of the fodder also has a regulating effect on the temperature. Obviously, a certain extant of moisture is necessary for the biological processes, but the half-dry plant parts get warmest, since heating of water claims a lot of heat.

Rapid increase in temperature up to 50° C. and more leads to sweet silage, since butyric acid bacteria do not develop so well at this temperature. Consequently, it was hoped in this way to arrive at a silage which would be well suited for dairy cattle. Unfortunately, however, even the sweet silage contains so many butyric acid bacteria, as was shown i.a. by BURRI, that it cannot be employed when the milk is used for Emmental cheese. There is still the possibility left completely to suppress butyric acid fermentation in the warm silage by inoculation with one of the thermophilic spore forming lactic acid producers which are closely related to hay- and potato bacteria. DEMETER (2) found such organisms in warm silage, and OLSEN (6) has shown that they play a part in the acidification of mash in distilleries, generally occurring at temperatures above 50° C. We have not made any experiments with these organisms in view of the fact that they only stand 0.5 % lactic acid and, therefore, scarcely will be capable of producing a pH in the silage sufficiently low to prevent the development of butyric acid bacteria. Furthermore, the proteins of the sweet silage seem to be hardly as digestible as those present in silage made at lower temperatures and, finally, it became clear that the vitamins are not so well preserved at the high temperature. It must be kept in mind that the main reason why green fodder is ensiled instead of being dried is to preserve its vitamins. This very significant fact has been emphasized by VIRTANEN (9); it is, however, amusing to point at the complete understanding of this circumstance long before the vitamins were discovered.

Already the ancient Greeks and Romans regarded cabbage as a medicinal plant which could heal many ailments. We know now that this is due to its high vitamin C content. As early as in 1739 [1]) the Hungarian army doctor HEINRICH KRAMER was fully aware of the important qualities of sauerkraut. He recommended that all ships should carry a certain provision of sauerkraut and that the men should have a portion of it every day in order to avoid scurvy. Thus, at that time he already realized that cabbage contains an anti-scorbutic substance which is preserved by acidification.

Our earliest investigations have shown that by spontaneous souring of any product the weaker acid-producers are gradually replaced by stronger ones: primarily are developed non-genuine lactic acid bacteria (coli-aerogenes bacteria; in milk, moreover, tetracocci), then genuine lactic acid bacteria, in the first place the spherical ones, subsequently the slowly growing, however

[1]) Already in 1720, the same doctor had shown that scurvy could be cured by means of lemon-juice (*cf.* (3)).

more strongly acidifying rod-shaped lactic acid bacteria. The acid reaction promotes the development of yeast and, in the presence of air, also the growth of moulds; the formation of lactates promotes the development of lactate-fermenting bacteria, such as propionic acid bacteria, butyric acid bacteria, and betabacteria. As shown by VAN BEYNUM and PETTE, the first mentioned bacteria cannot grow at pH lower than 5 and, as stressed by VIRTANEN, butyric acid bacteria grow poorly at pH below 4.2. The betabacteria are most acid resistant. Thus, E. OLSEN has found betabacteria in wine, growing at pH 2.75, which can stand 20 % alcohol (not yet published). In animal products such as milk and cheese, the spherical lactic acid bacteria are mostly streptococci, while they are generally betacocci in plant products such as beet slices, sauerkraut, and sour-dough. The rod-shaped lactic acid bacteria occurring in dairy products mostly are *Streptobacterium casei* and those found in plant products *Streptobacterium plantarum*. At higher temperatures the streptobacteria are replaced by thermobacteria.

In lucerne silage which is the only one considered in the present paper, only few betacocci were observed, while streptobacteria were predominant. Moreover, we have found a great deal of arabinose fermenting betabacteria, *i.e. Betabacterium caucasicum*, which presumably are identical with *Lact bacillus pentoaceticus* predominant in maize silage, as found by FRED and PETERSON (4). Also yeast, of course, is present in lucerne silage and contributes to the formation of alcohol, especially in the upper layers of the silage.

We have decided to use lucerne in our investigations, because this plant is particularly difficult to preserve in view of its high protein content and, hence, its strong buffer effect.

As already shown, i.a. by MØLLGAARD, plants such as cabbage and beet leaves, containing very little protein but much sugar, are easily preserved by spontaneous acidification, if only they are sufficiently chopped. However, crops rich in protein and poor in sugar cannot be preserved without addition of acid or sugar.

As is well known, the A.I.V. method is based upon the following procedure: the pH is immediately lowered below 4.2 by means of sprinkling with a mixture of hydrochloric and sulfuric acid during packing into the silo. The strong acids rapidly kill the plant parts which promptly shrink, and the loss by respiration becomes insignificant. On the other hand, these acids are very disagreeable. A splash into the eye may cause serious injuries and, moreover, the workers' footwear and clothes are ruined by these acids, while the silage is tramped together. Phosphoric acid, frequently used in U.S.A., is less corroding. Important extract substances get lost,

because as much as possible of the acid should be drained off prior to the neutralization of the silage with lime or soda. If the cows were fed non-neutralized silage, they would suffer from acidosis and, in the whole, it appears strange that they can stand the large amount of purging sulfates. At any rate, this cannot be called a natural food. Also in this respect it would be preferable to use phosphoric acid; and, moreover, in this case the neutralized drained liquid is of a high fertilizing value.

Therefore, much is in favour of the view that it will be better to add sugar in form of whey, molasses or Bergius' wood sugar (which, moreover, contains some biologically uninjurious hydrochloric acid). Unfortunately, in Denmark we can only work with whey and beet-sugar molasses. In order to prevent any loss of these precious substances, the silos should be completely tight so that no lucerne extract substances are lost either. As already shown by VAN BEYNUM and PETTE (1), so much sugar-containing liquid should be added that, together with the juice from the plant parts, it covers the whole mass and, thus, all air is driven out. The amount of sugar should be so large that lactic acid is formed enough to produce a pH below 4. For clover silage which was used by the Dutch investigators mentioned above, addition of 15 % whey with 0.5 % cane sugar proved to be sufficient. It will be shown later that this is not sufficient for lucerne.

Apart from the addition of sugar, it would be reasonable to inoculate with the right bacteria and, here, only streptobacteria can be taken into consideration. At ordinary temperature *Streptobacterium plantarum*, always occurring in silos, is the most vigorous lactic acid producer and, furthermore, it does not develop gas; thus, *Sbm. plantarum* does not cause any loss of dry substance or calories exceeding the 30 % which are lost in the conversion of sugar into lactic acid. This bacterium is extremely resistant and can be kept alive for many years without re-inoculation; much is in favour of the view that it is the progenitor of all other lactic acid bacteria, since plants are older than animals. Morphologically *Sbm. plantarum* appears as rods on solid substrates, while in liquid cultures it resembles streptococci. Other lactic acid bacteria are either rods or cocci. It forms equal parts of dextro-rotatory and levo-rotatory lactic acid, while most other lactic acid bacteria generally form merely one type of lactic acid. Of course, a more rapid development of the lactic acid fermentation and, thereby, a more effective suppression of the putrefying bacteria is obtained

when the total quantity of liquid added is acidified in advance. If the culture has pH 4, while the press-juice from the lucerne a pH of circa 6.5, the mixture will adjust itself to a pH of circa 5.3 which, as is known from our observations on cheese, inhibits putrefaction. Together with the cultures we thus add both sugar and acid, notably no inorganic acid which again has to be neutralized as in the A.I.V. method, but lactic acid, the caloric value of which can be utilized by the cows.

It was one of the aims of the present experiments to elucidate whether whey can be employed as silage solution, and we found that whey cannot be applied exclusively, firstly, because streptobacteria grow poorly in whey and, secondly, because it is impossible in this manner to supply sufficient amounts of sugar to the lucerne (*cf.* Table 4). However, addition of only 5 % molasses to the whey leads to a good substrate for streptobacteria. Supposing that the lucerne contains somewhat more than 80 % water with 0.7 % sugar, the whey 5 % sugar, and the molasses 50 % sugar, application of 20 % molasses whey relative to lucerne leads to just 2 % sugar in the juice. It is not convenient to add more than 5 % molasses to the cultures, since too high concentrations of sugar impede the growth of the bacteria. Thus, if more molasses should be used for the silage, the sugar must be added to the cultures after the bacteria have developed. If in this way the cultures contain 10 and 20 % molasses, the silage will contain 3 and 4 % sugar, respectively.

Earlier experiments have shown that both the rate of growth of streptobacteria and their acid formation are markedly increased by addition of a small quantity of manganese, *viz.*, 12.5 mg of $MnCl_2$. 4 H_2O per litre. Some examples will be given in the following. Table I shows the effect of increasing quantities of manganese on *Sbm. plantarum* 24 in milk. The numbers indicate $^0/_{00}$ of lactic acid formed.

As it appears from Table I, the maximum effect is already reached on addition of 10 mg of manganese salt to 1 litre of milk.

Corresponding results were obtained with all the nutrient substrates used by us, *viz.*, whey, casein peptone, yeast autolysate, and lucerne extract. This indicates that none of these substrates contains quantities of manganese worth mentioning. As soon as only 5 % beet-sugar molasses is added to the substrates, the acid formation is activated similarly, or more correctly, more vigorously than after addition of even 12.5 mg/l manganese salt; the activation

Table I.

mg $MnCl_2 . 4 H_2O$ per litre added	After 1 day	After 3 days	After 14 days
0	1.8	3.2	13.5
0.1	3.6	6.5	14.9
0.2	5.0	8.3	16.2
0.5	7.0	11.5	17.1
1.0	8.6	12.4	19.4
2.5	10.8	14.4	20.0
5.0	10.8	15.3	21.2
10.0	12.2	17.1	22.1
12.5	12.2	16.9	22.1
25.0	12.2	16.9	22.1
62.5	13.5	17.3	21.8
125.0	13.1	17.3	21.8

Table II.

Additions	After 2 days
None	2.9
5 % molasses	10.1
Manganese	7.9
5 % molasses + manganese .	9.9

is not further increased by addition of more Mn. This is illustrated by the following experiments (Table II) which again were performed with *Sbm. plantarum* 24 in casein peptone.

These experiments seem to indicate that beet-sugar molasses contains manganese, and an analysis actually proved that the manganese content of molasses is so high that addition of 5 % molasses just corresponds to the addition of the optimum amount of manganese.

The content of inorganic compounds of plants depending to a high degree on the content of these compounds in the soil, it might be a pure chance that in one single year the molasses of the Danish sugar manufactories contained so much manganese; however, in the molasses grown in other years we have found similar amounts. So we consider it permissible to conclude that addition of only 5 % molasses to the whey used makes a further addition of Mn superfluous. This holds especially in the case of lucerne which itself contains substances showing an activating effect on the growth of lactic acid bacteria (7).

If whey is to be applied as silage solution, such streptobacteria should be used for which milk is a natural nutrient substrate, *viz.*, not *Sbm. plantarum*, but *Sbm. casei*. In our silage experiments, we have therefore mainly worked with streptobacteria which are self-developing in lucerne with molasses whey without inoculation: No. 303 is such a streptobacterium. It grows rapidly in milk and dissolves casein in chalk-milk. It forms pure dextro-rotatory lactic acid and ferments neither melibiose nor arabinose. Thus, it is a typical *Sbm. casei*. As all streptobacteria it is strongly manganophilous, as will be seen from Table III.

Table III.

Sbm. casei 303	After 2 days	After 7 days	After 14 days
Without Mn	2.7	9.7	9.5
With Mn	10.4	17.3	21.6

Table IV shows the effect of molasses on whey and, further, the effect of lucerne extract on this mixture.

Table IV.

Sbm. casei 303	After 4 days
In whey	1.8
In molasses	3.6
In whey + molasses	9.9
In whey + molasses + 2 % lucerne extract . . .	10.8

For the silage experiment 500 g of lucerne, cut into small pieces, were stamped into high cylinder glasses; as described above, the lucerne has previously been mixed with 20 % bacteria culture in molasses whey without addition of manganese salt; the latter was made superfluous by the manganese content of the molasses.

In the first experiment, the developed gas was collected; later, however, we renounced this procedure, as we had made sure that the gas almost exclusively consisted of CO_2. Consequently, we confined ourselves to cover the silage with a board protected with a layer of solid paraffin and, moreover, charged with small stones and gravel. Owing to the CO_2-development it was unavoidable that some liquid was driven up over the board; therefore, the

uppermost 5 cm of the lucerne pile could not be applied to the analyses. Just as it is the case in big silos, the top layer which also became mouldy was of considerably minor quality than the remaining silage.

The cylinder glasses were kept at 30° C. either throughout the whole time of experiment or only in the beginning; subsequently, when the maximum temperature, *viz.*, 37° C., was reached, they were kept at room temperature. As a rule, 30° C. is the optimum temperature for streptobacteria (for *Sbm. casei* 303, however, the optimum temperature was 37° C.) and, therefore, storage at 30° C. promotes a rapid acidification but, in the long run, the silage cannot stand this high temperature. It was observed in practice that it is easier to make good silage in the autumn than in the summer when the silage is exposed to summer heat for several months. Under the prevailing conditions, the streptobacteria thrive fairly well at 15° C.; lower temperatures, however, should be avoided at the start of the silage. This can be done, even if the weather is not so warm, if the culture used is still warm.

The lucerne employed should not yet be in flower, but so young that juice is released during tramping. Since it is rather difficult to take a true average sample of the lucerne, we used for our analyses only the juice squeezed out by means of a Buchner press at 200 atm. pressure. We did not confine ourselves to the analyses of the juices before and after ensiling, we moreover de termined the raw-cellulose and the pentosanes of the press-cake in order to find out whether these substances had been converted during this process.

In the press-juice, the dry matter, the pH, the sugar content, especially pentoses, the lactic acid, acetic acid, and butyric acid as well as alcohol were determined. Among the N-containing constituents we differentiated between total N, pure protein N, amino N, and NH_3.

For the separation of the acetic acid and butyric acid, the only volatile acids present in the silage in measurable quantities, the partition method worked out by Osburn, Wood and Werkman (8) was applied. This method is as convenient as it is exact and is based on the different solubility of the acids in water and in ether.

From 100 ml press-juice 150 ml were distilled off in streaming water vapour. 50 ml of the distillate were used for a direct titration (N_2) and 50 ml for titration (N_1) after shaking in a separatory funnel for one minute with 50 ml ether. The ratio $K = \frac{N_1 \cdot 100}{N_2}$ is 69.3 for acetic acid, while it is 16.5

for butyric acid. Already a minute amount of butyric acid — which will distill first in a stream of water vapour — will considerably lower the given ratio.

Our first silage experiment performed on October 6, 1944, failed completely, because the lucerne was in flower and, thus, was too woody to be pressed together properly and to give any juice. Consequently, some air was present between the lucerne mass. The content of sugar was 3 %. Furthermore no streptobacteria had been isolated as yet from the lucerne with molasses whey and, therefore, we acidified with *Sbm. plantarum* 24. After the lapse of three months at 30° C., all lactic acid was converted into acetic acid and, owing to abundant development of ammonia, the pH had increased to 5.5. There were no considerable amounts of butyric acid, however, some alcohol was formed. It had an unpleasant odour.

In the following winter, when no fresh lucerne was available, we performed some experiments with dried lucerne which was soaked in water until it had assumed the same water content as fresh lucerne. We inoculated with *Sbm. casei* 303. The results were extraordinarily good both with 3 and 4 % sugar. This is certainly due to the fact that most bacteria normally present in lucerne were killed during drying so that, actually, we worked with a pure culture. After 5 weeks at 30° C., the pH still was 3.7. There had scarcely been formed any ammonia or volatile acids, but, practically spoken, all the acid formed was lactic acid.

On May 23, 1945, we started a more comprehensive series of experiments with fresh lucerne and 4 % sugar. During the first 3 weeks, the silage was kept at 30° C. and, subsequently, at 20° C. The investigations were performed after the lapse of 3 weeks and after 14—15 weeks. The following experiments were made:

1	No additions besides molasses whey	uninoculated
2	,, ,, ,, ,, ,,	inoculated with *Sbm. plantarum* 24
3	,, ,, ,, ,, ,,	,, ,, *Sbm. casei* 303
4	,, ,, ,, ,, ,,	,, ,, *Sbm. casei* 315
5	With addition of 1 °/₀₀ potassium bromate	,, ,, *Sbm. casei* 303
5*a*	With addition of 2 °/₀₀ potassium bromate	,, ,, *Sbm. casei* 303
6	With addition of 0.5 °/₀₀ potassium bromate	,, ,, *Sbm. casei* 315
6*a*	With addition of 1 °/₀₀ potassium bromate	,, ,, *Sbm. casei* 315
7	Lucerne killed by steaming . . .	,, ,, *Sbm. plantarum* 24
8		,, ,, *Sbm. casei* 303
9		,, ,, *Sbm. casei* 315

10 With addition of 0.5 % benzoic acid uninoculated
11 „ „ „ phosphoric acid „

If nothing but 20 % molasses whey were added, the uninoculated lucerne was essentially of poorer quality than the inoculated. Strange enough, the sugar disappeared more rapidly than in the inoculated lucerne. However, the pH did not decrease below 4, and the titre was lower than in the inoculated lucerne. This fact may be due to the somewhat stronger breakdown of the proteins. There were also formed greater quantities of acetic acid in the uninoculated than in the inoculated lucerne. The two *casei*-strains had almost the same effect nor exerted the *plantarum* strains any poorer action. All of them showed only a weak breakdown of protein and formation of acetic acid. The lactic acid formed both in the uninoculated silage and in that inoculated with *Sbm. planatarum* was mainly inactive, while in the two silages inoculated with *Sbm. casei* it was almost purely dextro-rotatory. This result indicates that inoculated bacteria can make their influence felt.

In order to decide whether the proteolytic enzymes which are effective during the ensiling are due to bacteria or to the lucerne itself, addition was made partly of an antisepticum, *viz.*, benzoic acid, which should injure the bacteria but not the plant enzymes, partly of bromate which, according to Holger Jørgensen (5), inhibits the effect of the papainases present in the plant cells. Finally, we destroyed them completely by steaming of the lucerne. The last mentioned procedure actually impeded the breakdown of proteins noticeably. In this experiment, however, neither bromate nor benzoic acid produced any marked effect on the proteolysis, nor did these chemicals hinder the development of streptobacteria. With bromate and *Sbm. casei* 303, the pH was still 3.9 in the 13th week, and with benzoic acid but without inoculation the pH was still 4.2 after 18 weeks.

As already mentioned, we did not use the A.I.V. method as a control for our silages, but we added pure phosphoric acid until pH 3.8. It must be admitted that here the breakdown of nitrogen was lowest. The phosphoric acid, however, could not impede the development of lactic acid bacteria.

On June 7, 1945, an experiment was started with different quantities of sugar, *viz.*, 2, 3, and 4 %, both inoculated with *Sbm. casei* 303 and uninoculated. The mass was first kept at 30° C. for 3 weeks, and at 20° C. during the remaining time. It was examined after the lapse of 6 months. Only the silage inoculated with 4 %

sugar proved to be good. It still had pH 3.9 and 0.77 % sugar unfermented. In all other cases, the sugar was completely fermented and the silage contained circa 10 times as much ammonia and 3 to 4 times as much acetic acid as the inoculated one. In the silage containing only 2 % sugar, both the inoculated and the uninoculated, the pH was above 5. A good deal of butyric acid had been formed. The ratio K was between 25 and 40.

The poor result can, however, partly be attributed to the fact that the lucerne layer was not high enough. Lucerne behaves just as a soft cheese which little by little ripens from outside inward due to moulds oxidizing the acid formed and bacteria producing ammonia which, gradually, penetrates into the mass. While the pH in the lucerne juice rapidly decreased below 4, it reincreased on the surface, and the spoiling processes set in.

Therefore, in the last experiment, we worked with cylinder glasses high enough to allow a lucerne pile of more than 30 cm, to which 750 g lucerne were applied. 3 or 4 % sugar were added (including the sugar content of the lucerne). Just as in an earlier experiment, we added bromate (2.4 g per kg) or phosphoric acid to pH 3.5. Moreover, experiments were performed with steamed lucerne. *Sbm. casei* 303 was exclusively used for inoculation. The press-juice was analyzed immediately and after the lapse of 5 months. Only in the beginning, the glasses were kept at 30° C., later at 20° C. It appears from Table V that the pH of the press-juice after addition of the acid culture decreased from 6.3 to 5.3 and that after 5 months (at any rate if inoculation was applied) it was below 4. The quantity of alcohol and volatile acids is smaller in the boiled lucerne, because yeast and most of the original bacterial flora are destroyed during the boiling process. The amount of volatile acid is greater in the uninoculated lucerne than in the inoculated, because foreign lucerne bacteria develop more vigorously in the former. This can also be concluded from the lactic acid formed which is more inactive [1]). Further, it is seen from the sugar content which decreases more rapidly in the uninoculated lucerne and thus no longer — as the source of acid it is — is able to counteract the neutralization from the surface. Consequently, more N has

[1]) For the determination of the optical properties of the lactic acid the zinc salts are prepared. αD = The rotation in the polarimeter for zinc salt of the dextro-rotatory lactic acid is —7 and the crystal water is 12.9 % while the crystal water content of the zinc salt of the inactive lactic acid amounts to 18 %.

The press juice of lucerne	Silage		pH	In 100 cc 0.1 n				Zink-lactate		% N as				%
Inoculation	% sugar content	Additions		Titer	alcohol oxidized to acetic acid	volatile acid	K 50/50	aD	% water	total	protein	amino acids	ammonia	sugar found
After 25 hours at 5° C.														
Uninoculated	3	0	6.3	44		6.0				0.29	0.10	0.19	0.01	3.1
Uninoculated	4	0	6.5	48		6.1				0.31	0.10	0.22	0.01	4.0
Inoculated	3	0	5.3	56		9.7		+ 6.0	12.9	0.24	0.06	0.18	0.01	
Uninoculated	4	phosphoric acid	3.5	395		1.0				0.19	0.05	0.14	0.01	0.72
After 5 months.														
Uninoculated	3	0	3.8	323	27.6	120.5	68	— 0.2	16.7	0.60	0.07	0.5	0.09	0
Inoculated	3	0	3.7	305	9.0	95.0	68	— 3.5	14.3	0.53	0.06	0.45	0.06	0.07
Inoculated	3	bromate	3.5	270	14.3	67.3	69	— 3.9	14.6	0.44	0.06	0.37	0.04	0.44
Boiled and inoculated	3	0	3.8	225	4.8	31.1	68	— 6.7	13.3	0.22	0.04	0.18	0.01	0.6
Uninoculated	4		4.1	356	18.7	94.0	69	0	17.8	0.64		0.61	0.07	0.14
Inoculated	4		3.8	365	16.8	29.5	65	— 5.6	13.9	0.57	0.06	0.41	0.06	0.68
Inoculated	4	bromate	3.8	345	19.3	30.3	69	—10.5	13.5	0.45		0.41	0.03	0.95
Boiled and inoculated	4		3.7	300	6.8	28.1	69			0.27		0.25	0.01	0.85
Uninoculated	0.7	phosphoric acid	3.1	430	47.1	20.2		— 0.3	16.9	0.38		0.29	0.003	0.05

been dissolved and more NH_3 was formed in the uninoculated than in the inoculated lucerne. Somewhat less volatile acid was formed with 4 % sugar than with 3 % sugar, however, in the present case, the quality of the inoculated lucerne was almost the same with 3 and with 4 % sugar. With effective protection against air and not too long storage, 3 % sugar is sufficient, *i.e.* addition of 2.3 % sugar, since the young lucerne itself contains circa 0.7 %. Butyric acid was not formed at all, otherwise K would have been markedly lower.

Just as in earlier experiments, also here the breakdown of nitrogen is inhibited by steaming of lucerne. Addition of bromate had also a favourable effect in this respect.

Addition of phosphoric acid to pH 3.5 impedes the breakdown of proteins in the same way as does steaming of lucerne. However, it is not able to suppress the lactic acid fermentation. The pH decreased further to 3.1, and in the juice many lactic acid bacteria were found, which will be studied more thoroughly. Nearly inactive lactic acid was formed just as in the remaining uninoculated lucerne. The amount of alcohol formed was relatively high. It was already observed by other investigators that neither the A.I.V. method can impede the lactic acid fermentation.

After having finished the laboratory experiments we performed an experiment on a somewhat larger scale at Virumgaard. Here, two silos were put at our disposal, consisting of big cement pipes, 96 cm in diameter and 200 cm high, on the inside lined with asphalt lacquer. Into each of these pipes were packed 400 kg of the same lucerne, treated only in a „ruffer", but not cut. To one portion were added 7.5 % of A.I.V. liquid and to the other portion 20 % of molasses whey, so that the juice contained 4 % sugar. After the mass had been covered with paper, a layer of 40 cm earth was put upon it. Not before a lapse of 16 days the A.I.V. silage had sunk from 182 cm to 102 cm, while the molasses whey silage already after 24 hours had shrunk from 156 cm to 100 cm. Owing to the abundant amount of liquid, the air was immediately expulsed, while long afterwards the A.I.V. silage contained some air which was never removed completely.

The ensiling was performed during the warmest summer season, from July 3, to September 10. On the 3rd of July, the air temperature was 23° C. and during the period of ensiling it rose to 28° C. In the A.I.V. silage the temperature decreased gradually from 28

to 16° C. In the molasses whey silage, however, after the second day the temperature increased owing to the fermentation heat to 29° C. and the high temperature was preserved for a longer time, until it finally sunk to 16° C. On the 10th of September, samples were removed from 30 cm depth and the A.I.V. silage smelled hardly as pure as did the molasses whey silage. We never met with A.I.V. silages which did not contain spores of butyric acid bacteria and, in the present case, we found more than in the molasses whey silage. The most important figures were as follows:

	pH	Titre	Volatile acid	K	NH_4-N
A. I. V. silage	3.81	98.5	138	58	0.07
Molasse whey silage .	3.51	160.0	44	69	0.00

The latter silage, reaching a pH below 4 in the course of 2 days, was therefore the best and, since no neutralization is needed before foddering and all the juice can be consumed, it is in all respects to be preferred to the A.I.V. silage. It is needed that cheese manufactories willing to produce the whey cultures are situated in the neighbourhood.

VIRTANEN's principle of adding acid to the silage is correct; however, if this is done by means of appropriate cultures of streptobacteria, it becomes superfluous immediately to acidify below pH 5.3, since hereby even the cheese mass is sufficiently protected against putrefaction. But, while the pH in solid cheese, owing to its poor sugar content, increases gradually to pH 6, the pH in silages rich in sugar will rapidly decrease below 4.

VAN BEYNUM and PETTE's principle, to add sufficient liquid in order to drive out all air, is also correct. If molasses whey is used as the sugar containing solution, cultures of *Streptobacterium casei* should be preferred to *Streptobacterium plantarum*; and if the fodder is so rich in nitrogen as is lucerne, also showing a strong buffering effect, 3 to 4 % sugar should be used (including the sugar content of the lucerne).

In concluding this paper, we want to give some examples of the fermentation capacity in casein peptone — yeast autolysate of the three most important ensiling bacteria, *Streptobacterium casei*, *Streptobacterium plantarum*, and *Betabacterium caucasicum*, later denoted as *Lactobacillus pentoaceticus* by FRED and PETERSON.

Since the latter denomination is most characteristic, we are willing to replace the name by *Betabacterium pentoaceticum*, especially in view of the fact that the bacteria occur more frequently in silage than in kefir grains.

The fermentation capacity is expressed in per mille lactic acid formed. As it will be seen from Tabel VI *Sbm. plantarum* found in lucerne silage, in contrast to *Sbm. casei*, can ferment arabinose (sometimes also xylose), raffinose and melibiose, but not inulin. It would appear natural that raffinose and melibiose fermentation always occur together, however,we have found some rare examples of raffinose fermentation without melibiose fermentation, and *vice versa*.

In the freshly isolated state, *Sbm. plantarum* grows poorly in milk, while *Sbm. casei* always acidifies the milk vigorously in the course of 3—5 days at 30° C. and also splits casein if the milk is neutralized with chalk.

Generally, the lactic acid formed by *Sbm. plantarum* is purely inactive, while that formed by *Sbm. casei* is dextro-rotatory or dextro-rotatory + inactive.

Betabacterium pentoaceticum is most peculiar, preponderantly fermenting arabinose and xylose, and in analogy with other pentoses fermenting lactic acid bacteria, into equal parts of lactic acid and acetic acid.The lactic acid is inactive.Generally, it moreover ferments glucose, sometimes also saccharose, however much more weakly than pentoses, and frequently it converts them almost exclusively into volatile products, as it was shown by FRED and PETERSON.

Since a not unappreciable amount of the sugar left after ensiling consists of pentoses, this can promote the growth of both *Sbm. plantarum* and *Sbm. pentoaceticum*. The main part of bacteria towards the end of the fermentation consisting of *Bbm. pentoaceticum*, there is reason to assume that the pentosanes are gradually dissolved. However, this does not apply to large amounts, and in the press-cakes from the silage we could even observe an increase in the amount of pentosanes from 12.7 to 14.5 % of the dry substance, because some proteins had been dissolved. For the same reason, the amount of cellulose can increase more pronouncedly (in poor silage from 23.6 to 48 %), but it is well known that raw cellulose is not broken down in an acid medium such as silage.

Finally, we wish to thank Dr V. STEENSBERG for numerous practical advices and Mr J. C. LUNDEN, director of the State Experimental Station, Virumgaard, who has the two silos packed for us and showed the greatest interest in the experiments.

No.	*Streptobacterium casei* isolated from:	Rotatory power of the lactic acid	Xylose	Arabinose	Sorbitol	Mannitol	Glucose	Mannose	Saccharose	Melibiose	Lactose	Raffinose	Inulin	Salicin	Time of curdling	Amount of acid
11	Emmental cheese	d	0	0.2	0.2	3.6	13.3	10.1	5.2	0.5	11.9	0	0.1	11.0	3	15.5
303		d	1.1	1.1	3.4	3.4	10.7	5.6	10.1	0.6	9.6	0	12.4	6.8	5	12.4
390	Lucerne	d	1.1	1.7	4.5	5.1	8.5	7.3	7.3	0	6.8	0	5.1	4.5	4	12.1
381	Silage		1.1	1.1	4.5	4.5	9.0	7.9	6.8	0	6.8	0	7.9	5.1	4	12.1
407	Uninoculated		0	0.6	4.5	4.5	8.5	8.5	10.9	0	8.5	0	10.1	5.6	4.5	13.8
493			1.1	1.1	3.4	2.4	9.6	5.6	9.0	0	8.4	0.5	10.7	6.8	3	14.2
	Streptobacterium plantarum															
24	Soured potatoes	i	12.6	12.8	2.9	2.9	8.3	8.8	9.2	7.0	8.6	8.6	0	7.9		2.9
333		i	5.6	13.0	4.0	4.0	7.3	4.5	4.0	5.5	6.8	5.6	0	4.5		3.2
343	Lucerne	i	2.8	8.5	1.1	4.0	4.0	4.0	3.4	6.0	5.1	5.6	0	4.0		2.2
444	Silage		2.4	14.1	0	0	10.1	9.0	9.0	6.0	0.6	6.5	0	7.3		0.9
465	Uninoculated		1.1	10.7	4.0	4.0	6.2	5.6	5.6	5.6	7.9	6.8	0.6	7.9		2.0
526			1.1	10.1	3.4	3.4	7.3	5.6	9.0	5.6	7.3	5.6	0	6.8		2.5
	Betabacterium pentoaceticum															
2	Kefirgrain	i	0.2	16.0	0	0	2.7	0	0	0	0.2	0	0	0		0
300	Cornsilage	i	8.7	9.7	0	0	3.8	0	0.5	0	4.5	0	0	0		2
379			6.2	5.1	0	0	4.5	0	0	0	0	0	0	0		0
447	Lucerne		5.6	13.5	0	0	2.8	0	0	0	0	0	0	0		0
490	Silage		7.9	10.7	0	0.6	4.5	0	0	0	0	0	0	0		0
510	Uninoculated		7.9	15.2	0	0	4.0	0	6.2	0	0	0	0	0		0
515			8.5	14.1	0	0	4.0	0	5.1	0	0	0.6	0	0		0

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(Molteno Institute, University of Cambridge).

COMPARATIVE STUDY OF SPORES AND VEGETATIVE FORMS OF *BACILLUS SUBTILIS*

by

D. KEILIN and E. F. HARTREE

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The factors which control the sporulation of vegetative forms of bacteria and the germination of spores are as yet undetermined. On the subject of sporulation extensive investigations have been carried out during the last 60 years and three different conditions have been suggested as governing this process: *a*) exhaustion of available nutrient material, *b*) accumulation of metabolic products and *c*) loss of water with consequent shrinking of colloids. The literature on this subject up to 1931 has already been reviewed by Cook (1).

Buchner (2, 3) working with *B. anthracis* concluded that spore formation resulted from an exhaustion of the nutrient medium. This work has been disputed on the grounds of incomplete controls and the view of Turro (4) that the accumulation of metabolic products induced sporulation was supported by Migula (5) who found that the spores began to appear at a certain density of population regardless of the size of the initial inoculum. Demnitz and Weyrauch (6), however, found that filtrates of old *B. anthracis* cultures had no effect on spore formation.

Williams (7, 8) working with *B. subtilis* found that growth was stronger on 5 % than on 1 % peptone but that spore formation took place more readily in the dilute medium. On the other hand, Cook (9) and Cook and Stephenson (quoted by Cook (1)) observed that in *B. subtilis* and in *Clostridium sporogenes* sporulation and active multiplication could proceed simultaneously. They believe that the predominance of spores in old cultures is due to lysis of the vegetative stages by the spores. Tarr (10), however, who investigated the growth of *B. subtilis* on media of various dilutions found, in agreement with Williams, that spores are formed in inverse ratio to the nutrient material available. Daranyi (11)

put forward the hypothesis that sporulation resulted from loss of water and shrinking of intracellular colloids. He concluded that in general similar conditions may favour both sporulation and multiplication.

Whereas the importance of the amount of available nitrogen has been disputed it is generally accepted that the presence of fermentable sugar inhibits the formation of spores owing to the accumulation of acid metabolites. In the case of aerobes oxygen accelerates the process of sporulation. Thus GREENE (12) in his studies of stained sections of bacterial colonies found that the sugar effect could be abolished by the addition of calcium carbonate and that spores were most numerous at the upper surfaces of cultures.

The marked heat resistance of bacterial spores has attracted considerable attention. TARR (13) found that spores of *B. subtilis* oxidize glucose after a short lag period and that previous heating to 80° C. increases the rate of oxygen uptake. However, once the actual oxidation of glucose by spores was under way it was as heat sensitive as the corresponding process in the vegetative cells and was abolished by heating to 50° C. It was also sensitive to cyanide and to narcotics.

VIRTANEN and PULKKI (14) failed to find any significant differences in chemical composition and water content of spores and vegetative cells of *B. mycoides*. FRIEDMAN and HENRY (15), however, were able to show that although the total water content of the two forms of *B. sub ilis* and of other bacilli is the same, the spores contain more „bound" water, *i.e.* water which cannot diffuse out into sucrose solution and so reduce the depression of the freezing point. ANGERER (16) unsuccessfully attempted to correlate the fat content and heat resistance of spores. An examination of the inorganic constituents of spores and vegetative cells of fourteen species of bacteria by CURRAN, BRUNSTETTER and MYERS (17) revealed that thermostability was associated with a high calcium content. Whereas spores contain more calcium than the vegetative forms, they contain less potassium and phosphorus.

The process of germination has, by contrast, received but scant attention. The process is invariably stimulated by transfer to a fresh medium and TOMKINS (18) showed that the humidity must be above a certain value. EIJKMAN (19) found that some salts inhibit the germination of spores while FILDES (20) observed that the

germination of *B. tetani* was stimulated when the redox potential of the medium fell below a critical value.

In this paper we propose to deal with a few biochemical properties of vegetative forms and spores of *Bacillus subtilis*. Special attention will be given to spectroscopic properties of cytochrome in vegetative forms and spores and to the manometric study of the processes of germination of spores followed by proliferation of the vegetative forms. The experiments summarized in this paper form part of more extensive investigations on certain biochemical aspects of sporulation and germination of bacteria; a study which began before the war and which suffered an unavoidable interruption. Although the account given here is far from complete we feel that even in its present state it may be helpful to other workers engaged in the study of various aspects of this interesting problem.

Material and methods.

The strain of the *B. subtilis* group used in the experiments described in this paper is No. 85 of the National Collection of Type Cultures, a strain known also as „Lister Institute, Hay 1916".

The organism was grown in Roux flasks upon the following medium: 10 g Lemco, 10 g peptone, 5 g NaCl, 30 g agar, and tap water to 1 L, the medium being adjusted to pH 7.3 with NaOH and sterilized for 20 minutes at 110° C. The flasks were inoculated from a stock spore culture preserved on the same medium.

To obtain vegetative forms, the flasks were incubated at 28° C. for 16 hours when the organisms, which were removed with great care so as to avoid contamination by the nutrient medium, were washed three or four times with distilled water in a centrifuge. When spores were required the cultures were kept at room temperature for 1—2 weeks until microscopic examination of smears, stained with fuchsin and methylene blue according to Mackey and McCartney (21), showed that spore formation was complete. The spores were washed three times and both spores and vegetative stages were stored in aqueous suspensions at 5° C. for periods up to 1 week. Spores were also stored in the dry state by mixing them with 10 volumes of alcohol, centrifuging, and drying the solid residue in a vacuum desiccator. After this treatment the spores remain viable for at least 2 years.

The respiratory activity of *B. subtilis* at different stages of its development was determined in Barcroft differential manometers at 37° C. The manometer flasks received the organisms

suspended in 3 ml fluid, and 0.3 ml 10 % NaOH plus a roll of filter paper was placed in the central tube to absorb CO_2. The experiments were carried out in 0.1 M phosphate buffer at pH 7.3.

Cytochrome in vegetative forms and spores.

According to previous observations (Keilin (22)), the vegetative form of *B. subtilis* shows a distinct 4-banded absorption spectrum of cytochrome when examined with a small dispersion spectroscope and a powerful light. The positions of the bands are approximately the same as those shown by baker's yeast, *Torula*, or the wing muscles of insects, namely: 605, 566, 550 and 521 mμ. The first three bands are the α-bands of the components *a*, *b* and *c* of cytochrome, while the fourth band is the combined β-bands of at least two components (*b* and *c*). Whereas the *B. subtilis* used in the present investigation (No. 85) showed this characteristic 4-banded absorption spectrum, two other strains of the *B. subtilis* group, nos. 2586 and 3610 of the National Collection of Type Cultures, were examined and found to differ from the strain 85 in having the bands *b* and *c* replaced by a single band (b_1) lying at 550—560 mμ. The absorption spectrum of cytochrome in these two strains therefore resembles that of *B. megatherium*. The fusion of bands *b* and *c* in the strain 3610 appears to be less complete than in the strain 2586.

Interesting results were obtained when the vegetative cells of strains 85 and 2586 were examined spectroscopically at the temperature of liquid air. At this temperature the absorption bands of reduced cytochrome became greatly reinforced, the degree of intensification being 5—7 times. Under ordinary conditions in strain 85, the band *c* is distinctly stronger than *b* but at liquid air temperature not only are the bands intensified but their relative intensities are reversed. Of especial interest was the observation that while the *b* and *c* bands are distinct both at room and at liquid air temperatures, there is an intermediate range of temperature at which they become fused. On the other hand, the fused band b_1 of strain 2586, which normally replaces *b* and *c*, is split into 3 bands when the cells are cooled in liquid air. One very sharp band lies at 554 mμ while two narrow faint satellite bands lie close to, and on either side of, this sharp band. With rising temperature this triplet is rapidly replaced by a strong single band which gradually fades to the normal intensity as the sample attains room temperature.

The cytochrome of the vegetative forms of all strains of the *B. subtilis* group behaves in the same way as that of yeast or of a preparation of washed minced heart-muscle. In presence of CO and of either a metabolite or a reducing agent the band *a* shows the characteristic widening towards the band *b* which is due to the formation of the a_3CO compound previously described (KEILIN and HARTREE (23)).

The cytochrome content of spores, unlike that of vegetative forms, is very low and usually imperceptible even on careful examination of a thick suspension of micro-organisms with a micro-spectroscope and a strong source of light. However, the absorption bands of cytochrome in spores become distinctly visible at liquid air temperature owing to the intensification already described. The relative cytochrome contents of spores and vegetative forms were estimated by determining the minimum depth of a suspension of each form which just showed the absorption bands of cytochrome. Thus the minimum depth of a spore suspension containing 26 mg dry weight per ml showing absorption bands at the temperature of liquid air was 3 mm, whereas the depth of a suspension of vegetative stages containing 19.6 ml was 1.5 mm at room temperature. Hence the ratio of cytochrome in spores and in vegetative stages is

$$\frac{1.5}{3} \times \frac{1}{6} \times \frac{19.6}{26} = 0.06.$$

The cytochrome content of spores is thus about 6 % of the cytochrome content of the vegetative cells.

The total haematin in the same two suspensions was estimated as pyridine haemochromogen. For this purpose 2 ml cell suspension was treated with 0.2 ml N NaOH and 0.4 ml pyridine. After addition of $Na_2S_2O_4$ the characteristic absorption spectrum of pyridine haemochromogen was observed. The intensity of this spectrum was compared with that of a standard solution of pyridine haemochromogen prepared from crystalline haemin, the determination being carried out with a microspectroscope and a double wedge trough as previously described (ELLIOTT and KEILIN (24), KEILIN and HARTREE (25)). The penetration of pyridine and reducer into the spores was very slow and the haemochromogen spectrum reached its maximal intensity only after freezing the suspension three times in liquid air. By this method it was shown that the haematin content of spores, expressed as per cent of dry weight, was 0.016 and that of vegetative stages 0.032. The spores

contain therefore an appreciable reserve of haematin which would be available during their proliferation for the synthesis of haematin catalysts such as cytochrome and catalase.

Resting metabolism of vegetative forms and spores.

The respiratory activity of *B. subtilis* was determined manometrically with thoroughly washed suspensions of the organism. Thus 1 ml suspension, containing 3.5 mg (dry weight) of freshly collected and washed vegetative cells, in presence of 1 % glucose or fructose took up 319 and 357 μl O_2 per hour respectively. Hence the Q_{O_2} at 37° C. is of the order 90—100. This oxygen uptake can be inhibited by cyanide, azide and by CO in the dark. The inhibition by CO, as shown in figure 1, is light sensitive.

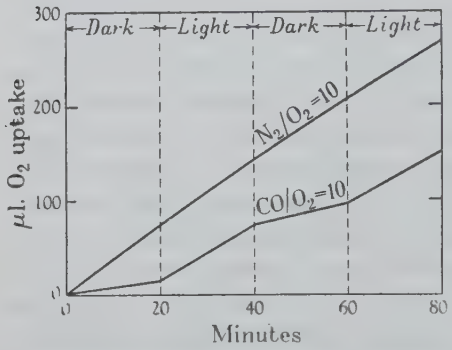

Fig. 1. Oxygen uptake of the vegetative form of *B. subtilis* (2.2 mg) in presence of 1 % glucose in gas mixtures $N_2/O_2 = 10$ and $CO/O_2 = 10$ and the effect of light from a mercury vapour lamp on the CO inhibition.

The Q_{O_2} of freshly washed spores under the same conditions was very low. After initial activation of the spores by heating to 80° C. for 30 minutes (Tarr (13)) the Q_{O_2} in presence of glucose was only 0.3 during the first hour but rose to a value of 3.9 during the second hour; a figure which was maintained for a further 4 hours. This oxygen uptake, although cyanide sensitive, is not affected by CO.

It must be pointed out that the Q_{O_2} values that we obtained for the resting metabolism of vegetative cells and spores do not agree with the figures given by Cook (9) and by Tarr (13). Their values for the vegetative forms are lower than ours but they found considerably higher activities in the case of spores than we were able to obtain even after several hours' incubation with glucose. On average we found the respiratory activity of spores to be only about 6 % that of fresh vegetative cells. The corresponding figures calculated from the results of Tarr and of Cook are 35—40 % and 90 % respectively. The only explanations of this wide divergence that we can offer are either that the previous workers made use of an entirely different organism or that their spore

suspensions contained sufficient of the nutrient media to initiate germination and proliferation during the experiments.

MANOMETRIC STUDY OF GERMINATION OF SPORES AND SUBSEQUENT PROLIFERATION OF VEGETATIVE FORMS.

The oxygen uptake of resting spores in presence of glucose was compared with that of spores to which were added certain amounts of yeast extract or of different nitrogenous compounds. The experiments were carried out with suspensions containing 1 mg spores per manometric flask in presence of 1 % glucose. The yeast extract (Kochsaft) was prepared by heating a suspension of 100 g baker's yeast in 200 ml distilled water for 30 minutes at 100° C. On filtering the mixture a clear yellowish solution was obtained containing 30 mg dry weight per ml.

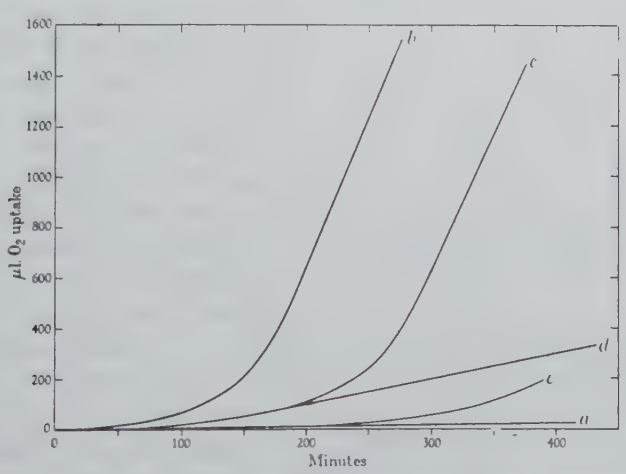

Fig. 2. Effect of yeast extract and 1 % asparagine on the germination of spores and the proliferation of *B. subtilis* in phosphate buffer containing 2 % glucose.
a) glucose only; *b*) glucose + 0.3 ml yeast extract; *c*) glucose + asparagine; *d*) glucose + 0.012 ml yeast extract; *e*) glucose + asparagine + 0.012 ml yeast extract.

The first series of experiments was devised in order to compare the effect of yeast extract with that of asparagine. The results of these experiments, which are summarized in Fig. 2, show that while the respiration of spores with glucose is very low (*a*), the addition of 0.3 ml yeast extract in a total volume of 3.0 ml causes germination within 2 hours and subsequent proliferation of the vegetative stages as indicated by the sharply rising curve *b* of

normal logarithmic growth. The rate of oxygen uptake eventually reaches a steady state, possibly owing to exhaustion of certain factors or metabolites concerned in the proliferation process. With smaller quantities of yeast extract (0.012 ml) the germination is complete as indicated by microscopic examination of the suspension at the end of the experiment, but a deficiency of nitrogen is now apparent which reduces or abolishes the proliferation (*d*). Thus the curves *a* and *d* show the comparative rates of respiration of equal numbers of spores and vegetative cells. Addition of extra nitrogen in the form of asparagine (1 %) gives rise to rapid proliferation in presence of 0.012 ml yeast extract (*e*) whereas asparagine alone causes only a very slow proliferation (*c*). Thus very small quantities of yeast extract will supply the factors necessary for germination of spores while larger quantities must be added, or alternatively other sources of nitrogen, in order to ensure growth and proliferation.

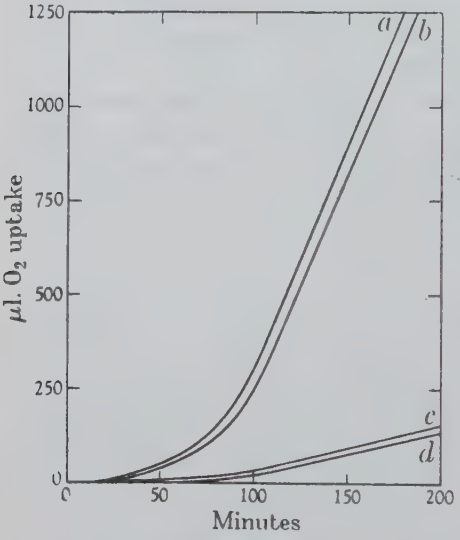

Fig. 3. Germination of *B. subtilis* spores (1 mg) in presence of glucose (1%) and different nutrient substances.
a) spores + glucose + tryptic digest of casein.
b) ,, ,, + yeast extract or peptone.
c) ,, ,, + dialyzed yeast extract.
d) ,, ,, + extract of boiled spores.

The extent of proliferation of the cells was determined by centrifuging the contents of the manometric flasks in special tubes, the lower half of which consisted of graduated capillaries.

In the course of these experiments, it was found that asparagine can be replaced by valine and arginine but not by histidine, tyrosine or glutamic acid.

After dialysis in cellophane tubes against distilled water, yeast extract is no longer effective (Fig. 3) which shows that the germination factor must be a comparatively small molecule. Moreover the factor is a very stable substance. Thus the dialysate from the above experiment was evaporated in a vacuum to a small bulk

and divided into 3 lots of 5 ml. The first lot was heated at 100° C. for 20 minutes with 0.5 ml N NaOH, cooled and neutralized with HCl. The second was similarly treated with 0.5 ml N HCl and neutralized, while the third was used as control. All three fractions induced germination and active proliferation. The germination of spores followed by active proliferation can be obtained with a tryptic digest of casein or with peptone in place of yeast extract. It is, however, important to note that yeast extract cannot be replaced by an extract of boiled spores (Fig. 3).

The viability of spores, as is well known, is not greatly affected by the process of drying in alcohol or by heating up to 80° C. This was demonstrated by taking three portions of freshly cultivated spores and incubating them for 15 minutes at 20°, 80° and 100° C. respectively. Three further portions were similarly prepared using spores which had been dried with ethyl alcohol and preserved 9 months in a desiccator over caustic potash. The oxygen uptake of these spores was measured in presence of glucose and yeast extract. The results, which are summarized in Fig. 4, show that whereas drying followed by heating to 80° C. does not markedly affect the spores, drying followed by heating to 100° C. greatly delays the germination and proliferation.

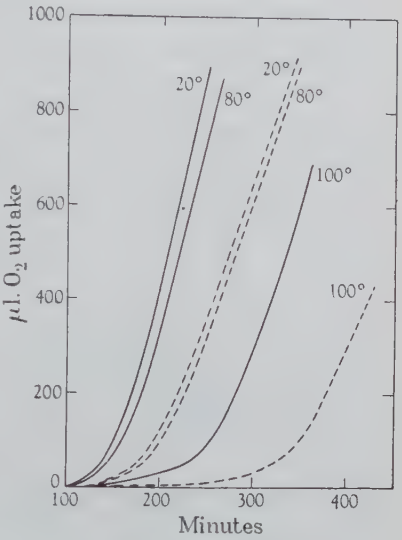

Fig. 4. Oxygen uptake by *B. subtilis* spores (0.4 mg) germinating in glucose (1 %) and yeast extract (0.3 ml). The spores were previously heated at 20°, 80° and 100° C. for 30 minutes. Full lines — spores freshly obtained; dashed lines — 9 months old, alcohol-dried spores.

Since the germination of spores is followed by multiplication of vegetative forms, experiments were carried out on the effect of yeast extract and other substances directly on freshly obtained vegetative forms of the organism. In these experiments (Fig. 5) yeast extract and peptone led to a very considerable increase in population of bacteria whereas dialyzed yeast extract, asparagine

and a mixture of amino acids (glycine, histidine, tyrosine, cystine, proline, asparagine, tryptophane, leucine, valine and arginine) gave no such increase, the extra oxygen uptake in such cases being due to an increased activity of the resting metabolism.

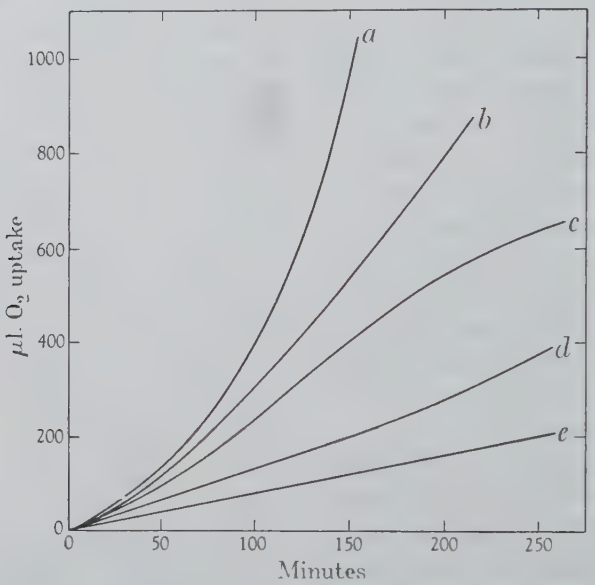

Fig. 5. Respiration of vegetative forms of *B. subtilis* (0.5 mg) in presence of glucose (2 %) and different nutrient substances.
a) glucose + 0.3 ml yeast extract; *b*) glucose + 0.3 ml 2 % peptone; *c*) glucose + 1 % asparagine; *d*) glucose + amino acids (3 mg each of glycine, histidine, tyrosine, cystine, proline, asparagine, tryptophane, leucine, valine and arginine); *e*) glucose only.

Effect of 8-hydroxy quinoline („oxine") on resting and proliferating metabolism of *B. subtilis*.

Whereas substances like cyanide, azide and iodoacetate which inhibit the respiration of vegetative stages inhibit also their proliferation in a nutritive medium, there are substances which inhibit proliferation without markedly affecting the respiration. Of these substances 8-hydroxy quinoline („oxine") and two of its derivatives will be considered in this paper.

A series of manometric experiments were carried out for this purpose with vegetative forms in presence of either glucose alone or glucose and yeast extract. Different concentrations of oxine or its dibrom- or dichlor-derivatives were added to the cell suspensions in the manometer flasks. The results of these experiments,

Table I.

Effect of 8-hydroxy quinoline (oxine) on resting and proliferating metabolism of vegetative stages of *B. subtilis* in phosphate buffer containing 1 % glucose.

a) Resting metabolism of 9 mg (dry weight) vegetative cells.
b) Resting metabolism of 2.7 mg vegetative cells.
c) Proliferating metabolism of 0.05 mg vegetative cells in presence of 0.3 ml yeast extract (y.e.).

Series	Medium	Oxine (molar)	O_2 uptake	% Inhibitor
			μl in 60 min.	
a	glucose	0	800	—
	,,	1×10^{-3}	552	31
	,,	2×10^{-4}	582	27
	,,	5×10^{-5}	730	9
	,,	2×10^{-5}	800	0
b	glucose	0	245	—
	,,	1×10^{-3}	241	2
			μl in 200 min.	
c	glucose	0	15	—
	glucose + y.e.	0	740	—
	,, ,,	2×10^{-4}	15	100
	,, ,,	5×10^{-5}	15	100
	,, ,,	2×10^{-5}	15	100

which are presented in Table I, show that in resting metabolism the inhibitory effect is marked only at oxine concentrations higher than 10^{-3} M. On the other hand, the proliferation of the vegetative forms is much more sensitive to oxine. The usual logarithmic increase of population in presence of 0.3 ml yeast extract is completely abolished by as little as 2×10^{-5} M oxine. Spore germination, as shown in Fig. 6, is also very sensitive to oxine. Thus 2×10^{-5} M oxine abolishes germination, a fact that is confirmed by microscopic examination, and this inhibitory effect disappears at a concentration of 2×10^{-6} M. Two derivatives of oxine, 5 : 7-dichlor- and 5 : 7-dibrom-8-hydroxy quinoline, were prepared according to the method of Berg (26) and tested with spores. The dibrom compound was approximately as active as oxine while the dichlor derivative was found to be more active, producing complete inhibition of germination at a concentration of 10^{-6} M.

That the inhibitory effect of oxine is perfectly reversible was demonstrated manometrically. For this purpose spores were incubated at 37° C. for 4 hours in presence of glucose and 10^{-3} M oxine in the flask of a differential manometer. The oxygen uptake

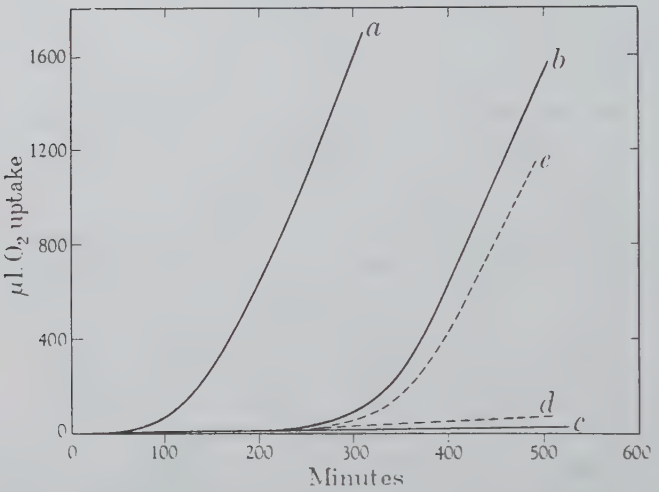

Fig. 6. Effect of oxine (8-hydroxy-quinoline) and its derivatives on respiration of spores (0.4 mg) of *B. subtilis* germinating and proliferating in 1 % glucose + 0.3 ml yeast extract.
a) control; *b*) + 6.6×10^{-6} M oxine; *c*) + 2×10^{-5} M oxine; *d*) + 1×10^{-6} M dichlor-oxine; *e*) + 2×10^{-5} M dibrom-oxine.

of the cells remained at the normal very low level during this time and no germination occurred. The cells were then centrifuged down, washed three times with water, and re-suspended in 3 ml phosphate buffer containing glucose and yeast extract. The oxygen uptake of this suspension followed the same logarithmic course as did a similar suspension which had not been in contact with oxine and the same degree of proliferation was observed in both cases.

Summary.

1. The vegetative forms of *B. subtilis* (N.C.T.C. No. 85) show a typical absorption spectrum of reduced cytochrome composed of 4 bands in the visible region belonging to the three components *a*, *b* and *c* of cytochrome. In presence of CO the band of the a_3CO compound appears as an extension of the short wave side of the band *a*. In certain other strains of the *B. subtilis* group the bands *b*

and *c* are replaced by a single band b_1 occupying an intermediate position.

2. Spores of *B. subtilis* show no absorption spectrum of cytochrome and only in suspensions cooled to the temperature of liquid air do the bands become visible. The cytochrome content of spores is about 6 % that of vegetative forms.

3. The haematin content of spores, estimated as pyridine haemochromogen, is about 50 % that of the vegetative forms. This shows that spores contain a sufficient reserve of haematin to build their cytochrome system during germination and proliferation.

4. The resting metabolism of vegetative forms at 37° C. in presence of glucose or fructose expressed in terms of Q_{O_2} is about 90. It is inhibited by cyanide, azide and by CO, the CO inhibition being light sensitive.

5. The resting metabolism of spores under similar conditions is very low; it increases somewhat after warming to 80° C. However, even warmed spores in presence of glucose show a very low rate of respiration, the Q_{O_2} being between 0.3 and 4. This oxygen uptake is cyanide sensitive but it is not affected by CO.

6. The germination of spores and the proliferation of the vegetative forms, which can be studied manometrically, require both assimilable nitrogen and an as yet undetermined factor present in yeast extract, peptone, or tryptic digest of casein. This factor cannot be replaced by an extract of boiled spores.

7. Most factors which inhibit the respiratory activity of these organisms also affect spore germination and subsequent proliferation.

8. There are, however, substances such as 8-hydroxy quinoline and its derivatives which do not, in concentrations 2×10^{-5} to 1×10^{-6} M, affect the resting metabolism of these organisms but inhibit almost completely both spore germination and proliferation of the vegetative forms. This inhibition is perfectly reversible.

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(From the Carlsberg Laboratory, Copenhagen).

THE SEGREGATION IN THE ASCUS OF *SACCHAROMYCODES LUDWIGII*

by

Ö. WINGE

(Received September 21, 1946).

As reported in a previous paper (WINGE and LAUSTSEN, 1939), in *Saccharomycodes Ludwigii* the spores are distributed in a characteristic manner. Even long ago it was observed that the 4 ascospores were lying in pairs, one pair in each end of the ascus. As a matter of fact, they adhere to one another so that it is only with some difficulty, that they may be separated by means of the micromanipulator.

In the paper mentioned above we were able to show that our material of *Saccharomycodes* — and thus, presumably, the species in general — was doubly heterozygous, *Nn Ll*, where *N* designates a gene for normal spore germination, while *n* signifies the alleomorphous gene for abnormal hypha-like germination that leads merely to the formation of a few cells before death appears. *L* is a gene for long growth of the cells, and *l* for short growth. Thus the doubly heterozygous ascus is able by meiosis to segregate either two spores of the formula *NL* and two of the formula *nl*, or two spores of the formula *Nl* and two *nL*. The probability is the same for either outcome.

It is a characteristic fact, however, that the two adjacent spores genetically are always the opposite of each other. Thus, in each end of the ascus there are always two spores of the formulas *NL* and *nl*, respectively, or two spores of the formulas *nL* and *Nl*.

Normally the adjacent spores always fuse at the germination, and hence the double heterozygote reappears in every case.

At a meeting in London, in the autumn of 1945, Professor J. B. S. HALDANE raised the question whether prereduction or postreduction took place here, as there had to be some particular explanation why the spores were always distributed in this way in the ascus.

This question may be answered to the effect that it must always be a matter of prereduction and, furthermore, that the two nuclei resulting from the first meiotic division continually — at the second meiotic division — give off one daughter nucleus to each pole. The latter feature was pointed out already in the work mentioned above, which thus was unable to confirm the view advanced by GUILLIERMOND (1903), as this investigator thought he had been able to demonstrate that two adjacent spores always contain sister nuclei.

The experiments with isolation of spores which we carried out previously, and on the basis of which the characteristic distribution of the spores was established, included the following observations on altogether 23 asci. Often germination of all four spores could not be obtained, but also the cases in which only three or two spores germinated afford some interesting information.

	One pair of spores	Other pair of spores
Ascus 1	*Nl n*	*Nl n*
,, 2	*Nl n*	*Nl n*
,, 3	*Nl n*	
,, 4	*NL n*	
,, 5	*NL n*	
,, 6	*NL n*	
,, 7	*NL n*	
,, 8	*Nl n*	*Nl n*
,, 9	*NL n*	
,, 10	*NL n*	*NL n*
,, 11	*Nl n*	*Nl n*
,, 12	*Nl n*	*Nl n*
,, 13	*NL n*	*n*
,, 14	*Nl n*	*Nl n*
,, 15	*Nl*	*Nl*
,, 16	*NL*	*NL*
,, 17	*NL n*	*NL n*
,, 18	*Nl*	*Nl*
,, 19	*NL n*	*NL n*
,, 20	*NL*	*NL*
,, 21	*NL*	*NL n*
,, 22	*NL n*	*NL n*
,, 23	*NL n*	*NL n*

As the spores containing the *n* gene do not reveal by their germination whether they contain *L* or *l*, since the germination appears

abnormal and soon ceases, it has not been practicable to give the complete formula for the *n* spores. But at any rate it can be established that in no instance were two *N* spores, two *n* spores, two *L* spores or two *l* spores found in the same end of the ascus. Furthermore, in all 16 cases where the *N* spores in both ends of the ascus could be analysed, they were found both to be either *NL* or *Nl*. Hence we are undoubtedly justified in concluding that also all *n* spores have differed genetically with regard to *L—l* from the adjacent spore. As was to be expected *L* and *l* spores are about equally frequent, the present observations giving 22 *L* spores and 17 *l* spores.

In the work mentioned above it was pointed out that the formula of the deviating *Saccharomycodes Ludwigii*-type is *NNLl*, and that this type must be a mutant or a result of fusion of an *NL* spore and an *Nl* from different asci. Therefore 6 asci have been examined in detail. As all the spores contain *N*, all 4 spores can be examined with reference to the *L* gene, provided the attempt to make them germinate turns out successfully. These 6 asci gave the following findings.

	One pair of spores	Other pair of spores
Ascus 1	*NL Nl*	*NL Nl*
„ 2	*Nl*	*NL Nl*
„ 3	*NL Nl*	
„ 4	*NL Nl*	*NL Nl*
„ 5	*NL Nl*	*Nl*
„ 6	*NL Nl*	*NL Nl*

As will be noticed here too we find in every instance a difference between the two adjacent spores with regard to *L*.

The explanation of this characteristic distribution of the genetically different spores is indisputable — and was advanced also by Lindegren (1945).

1) *N* and *L* are located in different chromosome pairs.
2) During the first division of the meiosis *N* is continually separated from *l*, and *N* from *n* (prereduction), as
3) both pairs of genes are situated so near the centromere that no crossing-over between this and the genes takes place.
4) At the 2-nuclei stage the nuclean spindles arranged themselves

parallel with the longitudinal axis af the ascus, so that both nuclei give off a daughter nucleus to each end of the ascus. This arrangement has been observed directly.

No doubt, the material here presented will justify these conclusions.

(Institut botanique de l'Université, Berne).

LA SPÉCIFICITÉ D'ACTION DU MÉSOINOSITOL, FACTEUR DE CROISSANCE POUR *EREMOTHECIUM ASHBYII*

par

W. H. SCHOPFER, TH. POSTERNAK et Melle M. GUILLOUD

(Reçu le 21 Septembre 1946).

Eremothecium Ashbyii Guill., très voisin d'*Ashbya gossypii* (Ashby et Nowell) Guill. (= *Nematospora gossypii* Ashby et Nowell), parasite du cotonnier découvert par GUILLIERMOND, est un Ascomycète synthétisant une forte quantité de lactoflavine diffusant dans le milieu. Les collaborateurs de GUILLIERMOND, Melle A. RAFFY, A. MIRIMANOFF ont étudié à l'aide de méthodes diverses cette flavine qui est bien la vitamine de croissance du groupe B_2.

Pour diverses raisons, nous avons été appelés à étudier à fond la physiologie culturale de ce microorganisme et à préciser le déterminisme de la flavinogenèse (voir SCHOPFER (9)).

Cet organisme est facilement cultivable sur un milieu naturel, à base de peptone par exemple. Ce type de milieu est le seul qui fut utilisé par GUILLIERMOND et ses collaborateurs. La première étape du travail consistait à cultiver le champignon sur un milieu synthétique. Nous y sommes parvenus (9). Les besoins en facteurs de croissance vitaminiques sont très voisins de ceux de *Nematospora gossypii*, étudié par KÖGL et FRIES (2). Sont requis pour un bon développement: la d,l-β-biotine, l'aneurine et le mésoinositol. Ces trois vitamines suffisent à *Nematospora gossypii*, mais pas à *Eremothecium Ashbyii*. Celui-ci requiert de plus une certaine dose d'un filtrat de peptone Witte, traitée par de la norite dans des conditions déterminées. A la suite de ce traitement, la peptone est privée, entr'autre de sa biotine et de son aneurine. Le filtrat seul est naturellement inactif. Il contient cependant un ou plusieurs facteurs absolument nécessaires à notre microorganisme. Le filtrat seul, et les vitamines seules sont inaptes au développement, dans les conditions de culture appliquées ici. Filtrat et vitamines agissent synergiquement.

Nous avons ensuite cherché à préciser la nature du ou des facteurs indispensables contenus dans le filtrat de peptone. Aucune des autres vitamines connues n'est active. Nous avons pu établir que le filtrat pouvait être remplacé par un mélange en proportions définies de d-arginine et de l-leucine (SCHOPFER et Melle GUILLOUD (10)). L'adjonction à ces deux acides aminés de l-histidine améliore encore la croissance.

Eremothecium Ashbyii est donc cultivable

1) sur un milieu synthétique additionné de peptone
2) sur un milieu synthétique additionné de d,l-β-biotine, d'aneurine et de mésoinositol et recevant en plus du filtrat de peptone
3) sur un milieu synthétique additionné de d,l-β-biotine, d'aneurine, de mésoinositol, de d-arginine, de l-leucine et de l-histidine.

Seul le troisième milieu est réellement synthétique, au sens propre du mot et tel qu'il est utilisable actuellement. Nous savons fort bien que le milieu dit synthétique, c. à d. constitué par des substances de composition chimique connue est une illusion et qu'il faut compter avec diverses impuretés introduites avec les constituants normaux du milieu, glucide par exemple. Nous pensons ici aux catalyseurs métalliques.

Remarquons encore que le milieu synthétique indiqué sous 3), conditionnant une bonne croissance, n'est pas celui qui détermine la plus forte flavinogenèse relative. Les conditions optimales des deux phénomènes ne sont pas identiques. Cet aspect du problème ne sera pas discuté ici.

Les trois vitamines requises n'ont pas toutes la même importance. La biotine est une facteur essentiel (en présence de filtrat). Seule, elle détermine un développement appréciable qui est amplifié par l'aneurine seule, ou le mésoinositol seul et surtout par l'ensemble aneurine + mésoinositol. L'augmentation de production de matière sèche, sous l'action du mésoinositol ajouté à la biotine et à l'aneurine est remarquable. Le mésoinositol seul n'exerce aucun effet. Il n'est donc que facteur complémentaire tout en ayant, à l'intérieur de la constellation, l'action quantitative la plus forte.

Ces raisons nous ont incité à étudier de plus près l'action de ce facteur de croissance. Les expériences qui font l'objet de ce mémoire ont toutes été effectuées sur un milieu de base identique: glucose 30 g, glycocolle 1 g, sulfate de magnésium 0,5 g, phosphate acide de potassium 1,5 g per litre d'eau distillée. A ce milieu nous ajoutons, par culture de 25 cc les vitamines requises: *d,l-β-biotine* 10 *mγ*, *aneurine* 10 *γ et mésoinositol* 1 *mg*. De plus chaque culture reçoit

0,5 ccm d'un filtrat d'une solution de peptone Witte à 6 %, traitée par la norite.

Les milieux sont stérilisées à 115° pdt 15 minutes, avec les vitamines.

Par le fait que le champignon se développe appréciablement en présence d'aneurine et de biotine, l'action de l'inositol ne peut, normalement, être appréciée que par la différence se manifestant entre les cultures aneurine + biotine et les cultures aneurine + biotine + mésoinositol.

L'idéal serait de trouver une condition culturale telle que le mésoinositol seul serait capable de déclancher la croissance, la biotine et l'aneurine, seules ou en combinaison étant incapables d'assurer la croissance. A première vue, ces exigences paraissent excessives. Le problème a été résolu de la manière suivante.

Toutes les peptones ne possèdent pas la même activité et ne sont pas toutes capables, seules et non traitées, de permettre la croissance d'*Eremothecium*. La peptone Witte, ainsi que la Bactotryptone Difco sont les meilleures. La Bactoprotone Difco est très peu efficace. Quoiqu'elle contienne 0,02 γ de biotine par gramme, elle est, seule, inutilisable par le microorganisme. Elle ne le devient que si on lui ajoute les trois vitamines, biotine, aneurine et mésoinositol, ou la biotine et le mésoinositol. La biotine seule est inactive. Le Tableau I indique le comportement du microorganisme en présence de bactotryptone et de bactoprotone.

Tableau I.

0	B	I	H	BI	BH	HI	BIH
Bactotryptone, 30 mg par culture de 25 ccm							
9,5 mg	12,5	9,4	9,9	21,9	12,0	9,6	23,6
Bactoprotone, 30 mg par culture de 25 ccm							
0,05 mg	0,15	0,70	0,50	0,60	0,70	12,6	17,3

B = 10 γ d'aneurine. I = 1 mg de mésoinositol. H = 10 mγ de biotine. Les chiffres indiquent le poids sec moyen de 5 cultures.

On voit nettement ce qui caractérise les réactions de l'organisme en présence de Bactoprotone. La Bactotryptone contient tout ce qui est nécessaire pour assurer le développement du microorganisme, aliments plastiques utilisables et vitamines. La bactotryptone seule fournit un poids sec de 9,5 mg, fortement augmenté par l'adjonction

de BI et de BIH. On est frappé par l'action très faible de la biotine ajoutée à la bactotryptone. Cela provient certainement du taux élevé de la bactotryptone en vitamine H, suffisant pour couvrir les besoins du microorganisme.

L'inactivité de la bactoprotone repose en partie sur sa faible teneur en vitamine, mais probablement aussi sur sa constitution. En effet, elle ne contient que 0,73 % d'acides aminés libres, alors que nous en trouvons 2,02 % dans la bactotryptone. Celle-ci est beaucoup plus fortement désintégrée que la bactoprotone.

En résumé, nous avons trouvé des conditions culturales excellentes pour étudier l'effet de l'inositol. Tous les milieux contiendront donc, à part les constituants indiqués ci-dessus, 30 mg de bactoprotone ainsi que la dose habituelle de biotine et de vitamine B_1. Les inosites sont ajoutés à la dose de 1 mg par culture de 25 ccm et, en général, stérilisés avec le milieu. Des contrôles ont été effectués avec les substances stérilisées à froid, par filtration sur Jena double.

Le mésoinositol agit quantitativement. A la dose de 1 γ par culture de 25 ccm, un effet peut être décelé. La dose optimale, dans les conditions culturales utilisées se trouve vers 150 γ. Avec 1 mg, nous sommes certains de pouvoir déceler une activité, si celle-ci doit se manifester.

Toutes les substances utilisées ont été préparées par l'un de nous (Posternak), qui a également établi leur constitution.

Le Tableau II indique l'activité des diverses substances, rapportées à celle du mésoinositol comptée pour 100. Nous avons étudié: le mésoinositol (6), le l-inositol (4), un inosose dit chimique préparé par oxydation nitrique du mésoinositol par Posternak (5), un inosose dit biochimique obtenu par l'action d'*Acetobacter suboxydans* sur le mésoinositol (Kluyver et Boezaardt(1a), Posternak(1a)), la scyllite (6), l'épi-inosite, la québrachite, éther méthylique de la l-inosite dont la position du groupe CH_3 n'est pas déterminée, la quercite (3), la mytilite (7), l'isomytilite (7), l'oxymytilite (7), l'oxy-isomytilite (7), un monophosphate d'inosite (1) ainsi que les trois cyclo-hexanes-triols 1, 2, 3 et l'hexaoxybenzol.

Dans l'appréciation des résultats quantitatifs, nous considérons le chiffre de 10, par rapport au mésoinositol, comme représentant la limite des variations normales. Seuls les chiffres supérieurs à 10 témoigneront d'une activité de la substance utilisée.

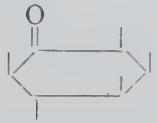
Nous constatons que deux substances, l'inosose biochimique (scyllo-méso-inosose) ainsi que le monophosphate d'inosite exercent

Tableau II.

1. méso-inositol = **100**

2. l-inositol = 10,4

3. inosose 1 racémique = 17,4

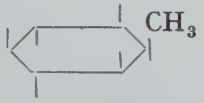


4. inosose 2 = **93,6**

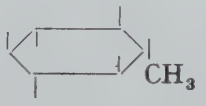
5. scyllite = 2,9

6. épi-inosite = 2,9

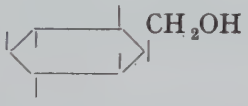
7. mytilite = 8,1



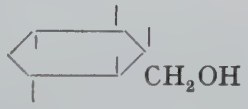
8. isomytilite = 19,7



9. oxy-mytilite = 7,5



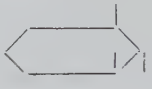
10. oxy-isomytilite = 2,9



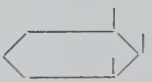
11. monophosphate d'inosite = **100**

12. québrachite = 22

13. quercite = 5,3

14. triol $\alpha = 3$ 

15. triol $\beta = 2,8$ 

16. triol $\gamma = 6,4$ 

17. hexaoxybenzol = 0

Pour simplifier, les atomes de H attachés au noyau ne sont pas indiqués.

la même activité que le mésoinositol. L'inosose chimique est faiblement actif, ainsi que la québrachite, fait singulier pour l'instant inexpliquable. La mytilite est inactive, alors que l'isomytilite l'est faiblement. La présence d'un groupe CH_2OH chez l'oxyisomytilite annule l'activité de la substance aux concentrations utilisées.

La spécificité d'action nous paraît donc assez marquée, et les résultats obtenus ressemblent beaucoup à ceux que nous avions obtenus avec *Rhizopus suinus* avec cette différence que l'inosose biochimique est très actif pour *Eremothecium Ashbyii* alors qu'il ne l'est par pour *Rhizopus suinus* (8).

D'une manière générale, l'activité vitaminique semble liée à la présence de trois groupes OH en cis. Pour l'inosose biochimique et le monophosphate d'inosite, nous pouvons admettre que l'organisme est capable de les transformer en mésoinositol. L'organisme ne peut réduire l'hexaoxybenzol en inositol. Il n'est pas impossible que l'activité du mésoinositol se manifeste par l'intermédiaire du métabolisme du phosphore.

Conclusions.

1. *Eremothecium Ashbyii* se développe sur un milieu contenant de la Bactotryptone non traitée. Sur ce milieu, l'aneurine, le mésoinositol et la biotine seuls sont peu efficaces. L'adjonction de mésoinositol à la constellation peu active aneurine + biotine, ou simplement à l'aneurine, détermine une forte augmentation du développement.
2. Il est confirmé qu'en présence d'un filtrat de peptone Witte ou de Bactotryptone, les trois vitamines sont indispensables pour que la croissance se produise.

3. L'adjonction au milieu de Bactoprotone, avec ou sans l'une des trois vitamines, ne détermine qu'une croissance extrêmement faible. L'adjonction de mésoinositol à la biotine, ou à la constellation biotine + aneurine, déclanche une très forte augmentation du développement. Dans ces conditions l'effet du mésoinositol et des substances analogues peut être analysée avec précision. Sur ce milieu, la spécificité d'action du mésoinositol est étudiée en détail.

   Ces expériences attestent une fois de plus l'importance de la composition du milieu lorsqu'il s'agit d'étudier ses réactions aux facteurs vitamines exogènes.

Les vitamines utilisées nous ont été aimablement fournies par le Département scientifique des Etablissements F. Hoffmann-La Roche & Co. SA (Bâle). Nous sommes redevables au Professeur KARRER de l'hexaoxybenzol utilisé.

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(From the Department of Physiological Chemistry, School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania).

NEW INVESTIGATIONS IN THE KINETICS OF CELL FREE ALCOHOLIC FERMENTATION

by

OTTO MEYERHOF

(Received September 8, 1946).

The disentanglement of the different steps of cell free fermentation of sugar to alcohol and CO_2 has captured the imagination of biochemists and microbiologists for a whole generation. It was also the topic of special interest to Professor KLUYVER.

Undoubtedly most of the laborious task of isolating the single steps was completed before the outbreak of the second world war. These results met with general acceptance from the scientific workers in our field. This is especially true for the sequence of about 12 stable intermediaries which lie on the pathway of this breakdown, as well as for the two coenzyme systems for oxidoreduction and phosphorylation. On the other hand the kinetics of fermentation contained some unexplained puzzles which took longer to unravel. Since these investigations published during the war are less well known, I may be allowed to summarize here three different studies which were published by me in the United States. These concern: The effect of arsenate (1); the origin of the Harden Young equation (2); the phosphate uptake during oxidation of glyceraldehydephosphate (3, 4).

1. THE ARSENATE EFFECT IN FERMENTATION.

The old discovery of HARDEN and YOUNG, that arsenate accelerates the fermentation of hexosediphosphate to the level of the fermentation rate of free sugar I ascribed earlier to a breakdown of the coupling between oxidoreduction and phosphorylation without alteration of the speed of this oxidoreduction (5). Thus hexosediphosphate or the 3-glyceraldehydephosphate formed from it by the „zymohexase" was rapidly converted into 3-phosphoglyceric acid without phosphate uptake. In the absence of arsenate

the same speed of oxidation of triosephosphate could only be obtained if simultaneously phosphate was taken up by adenosinediphosphate (ADP) with formation of adenosinetriphosphate (ATP). This ATP in presence of sugar transphosphorylates with hexose forming hexosephosphates (mono- and di-), while ADP is regenerated.

There was a second activation by arsenate, however, since 3-phosphoglyceric acid was dephosphorylated via phosphopyruvic acid in absence of phosphate acceptors with as high a speed as occurs in normal conditions only in the presence of phosphate acceptors.

Otto Warburg and W. Christian interpreted the first mentioned effect by the intermediate formation of 1-arseno 3-phosphoglyceric acid instead of 1,3-diphosphoglyceric acid which they had discovered to be the product of phosphate uptake in oxidation (6). While the latter acid transphosphorylates its acyl-phosphate with ADP, the arseno-compound splits its arsenate group spontaneously.

It can be shown (1) that this interpretation can be used also for explaining the second effect of arsenate. If no arsenate and no phosphate acceptors are present, phosphoglyceric acid decomposes mainly in the following way:

3-phosphoglyceric
↑ ↓
2-phosphoglyceric
↑ ↓

(1) phosphopyruvic + ADP → pyruvic acid + ATP

(2) ATP → ADP + phosphate

The reaction (2) depends on the presence of the ATP-ase, which is a very unstable enzyme in yeast.

By a side reaction the 3-phosphoglyceric acid decomposes partly also in the following way:

(3) ATP + 3-phosphoglyceric ⇄ 1,3 diphosphoglyceric + ADP

(4) 1,3-diphosphoglyceric → 3-phosphoglyceric + H_3PO_4
↑ ↓
phosphopyruvic

Here the diphospho-acid dephosphorylates spontaneously and the phosphopyruvic acid, which originates from the enzymatic equilibrium reacts further according to equation (1). No ATP-ase is used in this decomposition.

If arsenate is present a much quicker dephosphorylation than (4) occurs:

(5) 3-phosphoglyceric + ATP → 1,3 diphosphoglyceric + ADP
↓ (Dihydrocozymase)
3-glyceraldehydephosphate + phosphate

This reaction liberates continuously inorganic phosphate because in the presence of arsenate the oxidoreduction with cozymase is not truly reversible as in the absence of arsenate, but the reaction (5) is opposed by the oxidative step (6)

(6) 3-glyceraldehydephosphate + arsenate →
(cozymase)
1-arseno-3-phosphoglyceric → 3-phosphoglyceric + arsenate

While the triose-ester is oxidized by way of (6), phosphoglyceric acid is reduced by way of (5) and the net result is the liberation of inorganic phosphate from ATP, with formation of ADP. This ADP reacts again with phosphopyruvic according to equation (1).

This sequence of reactions could be definitely established by application of NaF which blocks the ,,enolase'', the reversible transformation:

(7) 2-phosphoglyceric ⇄ phosphopyruvic

By this blocking the arsenate effect is abolished.

2. The origin of the equation of Harden-Young.

The stoichiometric accumulation of hexosediphosphate + hexosemonophosphate in cell free fermentation, discovered by Harden and Young (7) was for a long time a great puzzle because such an accumulation is not observed with living yeast, although some ester accumulates here too (8).

The origin of the Harden Young equation has to be looked for in the coupling of oxidation with phosphate uptake, in the reaction of glyceraldehydephosphate to 1,3-diphosphoglyceric in the presence of phosphate and cozymase. Subsequently the diphosphoglyceric acid transfers its labile acylphosphate to the adenylic system, the ATP formed in this way phosphorylates hexose to the hexosephosphates. The triosephosphate formed by the ,,zymohexase'' reaction from hexosediphosphate, reacts again in the same manner; but after the phosphate acceptors are exhausted or all inorganic phosphate removed, the triose-ester can be oxidized only in the way discussed above. This means that ATP formed in the coupling reaction can be split in larger amounts only by the ATP-

ase. From this connection follows the inference that the accumulation of hexosediphosphate is due to a relative lack of ATP-ase. The living yeast, in which the same reactions would occur, must contain enough ATP-ase for keeping pace with phosphorylation. The Harden Young equation therefore results from destruction of ATP-ase during drying and extracting the yeast.

All these assumptions were proved with cell free extract from yeast prepared either by ultrasonic vibration or by freezing in liquid air. The following results were obtained: (a) The fermentation rate of hexosediphosphate in the different preparations is exactly proportional to their content of ATP-ase. (b) ATP-ase is strongly adsorbed on the structural elements of the cells and extremely labile before purification. (c) By adding a solution of concentrated ATP-ase from potatoes to yeast extract, the fermentation rate of hexosediphosphate is raised to the highest level, the ,,arsenate level'' above the rate of fermentation of free sugar. (d) If potato enzyme is added to a yeast extract fermenting free sugar at a time where the speed of the phosphate period has fallen down to the low level of the second period, the rate rises again to the level of the phosphate period. By mere addition of enough purified ATP-ase the fermentation type of yeast extract is changed into the fermentation type of living yeast.

3. Phosphate uptake during oxidation of glyceraldehyde-phosphate.

Inorganic phosphate is taken up during fermentation in two places, in the reversible phosphorylation of glycogen (9), the ,,phosphorolysis'' with formation of glucose-1-phosphate, and secondly in the oxidation of glyceraldehydephosphate.

Warburg and Christian assumed that a precursor of the 1,3-diphosphoglyceric acid would be formed first, 1,3-diphosphoglyceraldehyde with a carbonylphosphate similar to that of Cori's ester.

All attempts, however, have failed to demonstrate the presence of such a compound. Neither the equilibrium between the dihydroxyacetone phosphate and 3-glyceraldehydephosphate nor that between triosephosphate and hexosediphosphate is influenced by high concentrations of phosphate. Especially the ,,isomerase'' equilibrium of triosephosphate, giving a distribution of 4 to 4.5 % aldotriose to 96% keto triose is not changed even by .1 to .2n inorganic phosphate (3, 4).

On the other hand with cozymase and the oxidizing enzyme, in presence of .1 to .15n inorganic phosphate the reacting concentration of glyceraldehydephosphate is about 25 % to 50 % above the calculated distribution, that is about 5 to 6 % aldotriose, if isomerase, or isomerase and aldolase are present and can reestablish the equilibrium conditions when part of the glyceraldehydephosphate is oxidized to 1,3-diphosphoglyceric acid.

It is probably that a loose addition product is formed between the 3-glyceraldehydephosphate and inorganic phosphate in presence of the oxidizing system. Only by oxidation is this addition product transformed into a chemical compound.

A similar situation exists apparently in the enzymatic system of *Bacterium Delbrücki*, where pyruvic acid in presence of phosphate and flavin enzyme is oxidized to acetylphosphate according to F. Lipmann (10). The true mechanism of the oxidative formation of these acylphosphates remains to be established.

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(Laboratoire de Chimie biologique, Institut de Chimie de l'Université, Lyon).

SUR LA RESPIRATION DU CILIÉ *TETRAHYMENA GELEII*

par

PAULETTE CHAIX, JULES CHAUVET et CLAUDE FROMAGEOT

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L'étude du système respiratoire des protozoaires, si importante du point de vue de la physiologie cellulaire comparée, n'a commencé à être entreprise que depuis un petit nombre d'années. Le principal écueil était l'impossibilité où l'on se trouvait d'obtenir ces organismes en culture rigoureusement pure et en milieu liquide. Cette difficulté ayant été surmontée pour quelques espèces de protozoaires (13), il est devenu dès lors possible de mesurer la respiration de base de ces organismes en fonction de l'âge des cultures, de la température, du pH, de la composition du milieu minéral et de la nature du substratum éventuellement ajouté. Mais ces données fondamentales élémentaires sont encore loin d'avoir été aussi bien précisées que pour certaines levures, certaines bactéries ou certains tissus, et un énorme travail expérimental reste à faire, en particulier en ce qui concerne la détermination des substrats qui, à une concentration déterminée, doivent permettre au système respiratoire de ces protozoaires de fonctionner avec son activité maximum.

C'est à la recherche de ces substrats préférentiels qu'ont été consacrées les expériences décrites dans le présent travail, pour le cas d'une variété de ciliés bien définie, cultivable en milieu liquide, en l'absence de toute contamination bactérienne. Il s'agit d'un cilié qui nous a été aimablement fourni par A. Lwoff sous le nom de *Glaucoma piriformis* [1]) (10) et que Furgason (4) considère comme devant être désigné sous le nom de *Tetrahymena geleii*. Nous étudions ici la respiration de ce microorganisme à l'état non proliférant en milieu minéral simple, tamponné, additionné ou non d'un substrat organique.

[1]) L'orthographie piriformis ou pyriformis a fait l'objet de discussions (4). Signalons que le mot dérive du latin pirum, i, ou pyrum, i; les deux manières d'écrire sont donc indifféremment acceptables.

Pr paration du microorganisme.

Tetrahymena geleii se cultive aisément en grande quantité dans un milieu liquide stérile, constitué par: peptone 15 g; NaCl 3 g; eau de levure simple [1]) 2,5 cm³; eau bidistillée q.s. pour 1000 cm³. La réaction du milieu doit être ajustée à pH 7.0 à 7.2. La température optimum de culture est comprise entre 20 et 25° C. (8).

L'intensité de la respiration de *Tetrahymena geleii*, mesurée aussi bien dans le milieu de culture initial que dans un milieu minéral simple tamponné, dépend de l'âge des cultures (1, 12). Ormsbee (12) a précisé que cette intensité est maximum pendant la période exponentielle de la croissance, soit entre 20 et 30 heures après ensemencement. Les organismes utilisés dans le présent travail ont été récoltés dans tous les cas 70 heures après ensemencement, c'est-à-dire au cours de la phase stationnaire.

Etant donné la fragilité des organismes en question, la récolte nécessite certaines précautions: le milieu de culture est centrifugé 100 secondes à 1000 t/m (R = 20 cm); le culot de centrifugation est mis en suspension dans la solution minérale suivante: NaCl 0,666 g; KCl 0,094 g; SO_4Mg. 7 H_2O 0,284 g; PO_4Na_2H. 12H_2O 7,85 g; HCl N 4,85 cm³; eau bidistillée q.s. pour 1000 cm³. Cette solution est à pH 7.2 à 7.3. La suspension ainsi obtenue est passée sur un filtre de nylon placé sur un entonnoir de Büchner; elle est ensuite centrifugée une seconde fois dans les mêmes conditions, puis le culot est remis en suspension dans la même solution physiologique, et la nouvelle suspension est filtrée. Cette dernière suspension convenablement diluée est utilisée pour les mesures de consommation d'oxygène. De plus nombreux lavages par centrifugation ne sont pas possibles car ils endommagent sérieusement les cellules. Avant chaque expérience, on contrôle au microscope l'état physiologique des ciliés. La suspension n'est utilisée que si le nombre des cellules mortes est négligeable, et si les cellules vivantes présentent une motilité normale. La concentration finale en ciliés de la suspension oscille entre 100 et 400 par mm³.

Disposition des expériences.

Les mesures de consommation d'oxygène sont effectuées sur un volume de 2 cm³ de la suspension de ciliés à l'aide de la technique manométrique de Warburg; le CO_2 est absorbé par une solution

[1]) L'eau de levure simple est préparée en autoclavant une suspension de 100 g de levure pressée dans 1000 cm³ d'eau, pendant 3 heures à 120° C., et en décantant puis filtrant jusqu'à obtention d'un liquide limpide.

de potasse à 30 % placée dans le récipient central selon la méthode habituelle. Les mesures sont faites, sauf indication contraire, à 22° C., à pH 7.3 et en présence d'air.

Expression de la consommation d'oxygène.

Il convient de définir nettement l'unité de matière vivante à laquelle on doit rapporter la consommation d'oxygène observée dans l'unité de temps.

L'usage général de choisir comme unité le mg de poids sec se heurte ici à des difficultés:

Le poids de sels minéraux dissous dans la suspension étant très grand par rapport au poids sec des microorganismes contenus dans un même volume, la détermination de ce poids sec par simple différence entre une pesée de la solution minérale et une pesée d'une même volume de suspension, est peu précise. Pour obvier à cet inconvénient, certains auteurs centrifugent aussi complètement que possible les ciliés puis les lavent à plusieurs reprises avec de l'eau distillée et déterminent le poids sec des cellules ainsi lavées (12). Cette manière de faire n'est pas exempte de critique, car l'eau distillée est capable de lyser une partie des ciliés, et parce que les centrifugations énergiques indispensables font que le protoplasma de ciliés s'agglomèrent en un gel difficile à mettre en suspension homogène et difficile à laver.

M. Lwoff (9) rapporte la consommation d'oxygène au poids sec. Mais d'après ce qu'a bien voulu nous dire cet auteur ce poids sec est déterminé de la manière suivante: 5 à 10 cm^3 de suspension de ciliés sont additionnés de 2 à 4 gouttes de formol à 30 %; il se forme un précipité; ce précipité est centrifugé, lavé à trois reprises avec de l'eau bidistillée puis sèché et pesé. Le poids sec ainsi obtenu est considéré par M. Lwoff comme l'équivalent du poids sec direct des microorganismes. Cette manière d'opérer est a priori séduisante. Cependant, un contrôle nous a montré qu'elle donne des valeurs de poids sec très inférieures à celles du poids sec direct. Le Tableau I permet de comparer le poids sec des microorganismes calculé à partir de l'azote total, au poids sec expérimental des microorganismes traités par le formol. Le tableau permet également de comparer l'azote total de la suspension de microorganismes à l'azote total du précipité obtenu en traitant par le formol un même volume de la suspension. Il met en évidence la présence d'une forte quantité d'azote dans les eaux de lavage du précipité, ce qui indique une perte de substance dans ces eaux de lavage. Il est à noter que les

écarts entre „poids sec réel" et „poids sec formol" sont beaucoup plus accentués dans le cas d'une suspension de ciliés faite dans le liquide physiologique tamponné utilisé ici, que dans le cas d'une suspension de ciliés dans le liquide dit „Ringer pour batraciens", utilisé par M. Lwoff (9).

Tableau I.

Etude quantitative du précipité obtenu à partir d'une suspension de *Tetrahymena geleii* après addition de formol et lavage.

Les chiffres sont exprimés en mg.

I = Suspension de *Tetrahymena geleii* dans la solution dite milieu de Ringer pour batraciens (9).

II = Suspension de *Tetrahymena geleii* dans la solution tampon utilisée dans le présent travail.

	I	II
Poids sec calculé à partir du dosage de N total	11,0	8,6
Poids sec expérimental du précipité obtenu après addition de formol	3,0	3,4
Azote total dosé dans la suspension de cilié mise en expérience	1,190	0,865
Azote dosé dans le précipité au formol . . .	0,250	0,246
Azote dosé dans les eaux de lavage du précipité par le formol	0,780	0,591
Azote total retrouvé	1,030	0,837

D'après Ormsbee (12) la teneur moyenne en azote total de *Tetrahymena geleii* est de 10,5% du poids sec; d'après le présent travail (Tableau II) elle est de 10,8 %. Le poids sec dont il s'agit dans le Tableau I est calculé à partir de la moyenne de ces deux chiffres, soit 10,6 %.

Les irrégularités des chiffres du Tableau II tiennent au manque de précision de la détermination du poids sec.

On peut d'autre part rapporter la consommation d'oxygène à un nombre de cellules donné. Ceci nécessite la numération des ciliés, opération aisée et relativement précise. Certains auteurs (1, 5, 12) préconisent cette méthode, qui est correcte pour les organismes récoltés à la phase stationnaire de la croissance, et dont la taille est pratiquement constante ($50\,\mu \times 20\,\mu$). Cette méthode n'est par contre pas utilisable si les ciliés sont récoltés dans la phase exponentielle de leur croissance, phase durant laquelle la taille individuelle des ciliés peut être variable (12).

Enfin, on peut choisir comme unité de référence le mg d'azote

Tableau II.
Teneur en azote total de *Tetrahymena geleii*.

Poids sec direct (mg)	N total (mg)	N total %
3,44	0,288	8,4
5,80	0,494	8,5
3,30	0,408	12,0
4,50	0,511	11,0
3,36	0,420	12,5
3,60	0,425	11,8
4,00	0,394	10,0
1,90	0,210	11,0
2.04	0,250	12,2
	Moyenne:	**10,8**

Tableau III.
Respiration de base de *Tetrahymena geleii*.

T = 22° C.; pH = 7.3; chaque expérience est faite avec une même récolte de ciliés, et comporte plusieurs déterminations, dont le nombre est indiqué entre parenthèses.

Azote en γ/2 cm³ de suspension de cilié	Q_{O_2} première heure d'expérience	
420	15,2	(4)
72	16,3	(6)
425	16,0	(12)
394	17,2	(10)
116	11,1 [1])	(4)
159	18,3	(2)
200	13,9	(9)
196	13,2	(11)
127	11,2	(12)
130	13,3	(4)
119	12,5	(12)
210	13,0	(10)
142	13,4	(5)
205	14,8	(12)
155	16,4	(12)
120	13,3	(12)
200	13,1	(12)
203	14,6	(4)
Moyenne:	**14,3**	

[1]) Les ciliés de ces essais ont été cultivés sur eau peptonée glucosée 0,5 %, et non sur eau peptonée simple.

total directement dosé dans la suspension de ciliés mise en expérience. Ce mode d'expression paraît le plus rigoureux car il permet de rapporter le consommation d'oxygène à l'unité de protoplasma. C'est celui que nous avons adopté ici. Mais pour nous conformer à l'usage d'exprimer l'intensité de la respiration par Q_{O_2} = mm³ O_2/mg poids sec/heure, nous calculons le poids sec des cellules à partir de l'azote total en admettant que cet azote représente 10,6 % du poids sec.

Respiration de base de *Tetrahymena geleii*.

Le Tableau III résume les résultats obtenus au cours d'une série d'expériences destinées à la mesure de la respiration de base de *Tetrahymena geleii*.

Les chiffres de ce tableau montrent que dans les conditions réalisées, à une température de 22° C., Q_{O_2} moyen = 14,3, avec des écarts ne dépassant guère ± 25 %. Quelques expériences faites à la température de 25°2 C. donnent une valeur moyenne Q_{O_2} = 19,1.

Si l'on compare ces chiffres à ceux obtenus par d'autres auteurs

Tableau IV.

Vitesse de la respiration de *Tetrahymena geleii* en cultures pures d'après différents auteurs.

Auteurs	Age approximatif (heures)	Solution dans laquelle sont mis en suspension les ciliés	pH	T°	Q_{O_2}
		a			
M. Lwoff (9) . .	150 [1]	+ glucose 0,1% [1]	?	22	35 [2]
E. G. S. Baker-J. B. Baumberger (1)	48	b + glucose 0,5%	7	20-22	19,6
R. A. Ormsbee (12)	50	c	6.9	26.8	16,5
Expériences personnelles	70	d	7.3	22	14,3
				25.2	19,1

a = NaCl 6,75; KCl 0,15; $CaCl_2$ 0,2; eau bidistillée 1000 cm³.
b = autolysat de levure à 10 % (1).
c = tampon phosphate 0,005 m.
d = solution décrite p. 146.

[1]) D'après une communication personnelle.

[2]) En tenant compte du fait que le poids sec du précipité par le formol est environ 3,5 fois plus faible que le poids sec réel; Q_{O_2} calculé comme dans le présent travail serait donc environ égal à 10.

travaillant avec *Tetrahymena geleii* en culture pure et dans des conditions définies, on constate une certaine homogénité des résultats (Tableau IV).

ACTION DU BUTYRATE DE SODIUM SUR LA RESPIRATION DE *Tetrahymena geleii*.

Une première série d'expériences, dont le détail est donné dans les Tableaux V et VI, montrent que le butyrate de sodium accélère de façon notable, 54 % en moyenne pour une concentration 0,01 M, la consommation d'oxygène par *Tetrahymena geleii*. Cette accélération, optimum pour 0,01 M, est moins sensible pour des concentrations supérieures.

Tableau V.

Influence du butyrate de sodium 0,01 M sur la respiration de *Tetrahymena geleii*.

A = Q_{O_2} calculé pour l'heure précédant l'introduction du butyrate.
B = Q_{O_2} calculé pour la deuxième heure suivant l'introduction du butyrate, dans les essais réels.

Les valeurs correspondant aux essais témoins ne recevant aucun substrat d'un bout à l'autre de l'expérience sont désignées par la lettre T. Les chiffres entre parenthèses indiquent le nombre de déterminations.

N° de l'expérience	Azote en γ/2 cm³ suspension *Tetrahymena geleii*	A	B	Accélération brute	Accélération corrigée par rapport au témoin
460318	200	12,7	19,1	+ 50 %	+ 50 %
		13,8	19,3	+ 40 %	+ 40 %
		11,2 T	11,2 T	0	—
460325	196	12,7	18,8	+ 47 %	+ 59 %
		13,2 T	11,7 T	— 12 %	—
460401[1])	127	11,1 (2)	17,7	+ 59 %	+ 52 %
		11,3 T (2)	12,1 T (2)	+ 7 %	—
460408	130	13,2 (2)	22,0 (2)	+ 66 %	+ 76 %
		13,5 T (2)	12,2 T (2)	— 10 %	—
460429	119	16,2 (2)	20,4 (2)	+ 25 %	+ 46 %
		15,3 T (2)	12,0 T (2)	— 21 %	—
460506	210	13,5 (2)	15,7 (2)	+ 17 %	+ 44 %
		12,3 T (2)	9,0 T (2)	— 27 %	—
460513	142	13,2 (2)	18,1 (2)	+ 37 %	+ 64 %
		13,2 T (2)	9,6 T (2)	— 27 %	—
				Moyenne:	+ 54 %

[1]) Butyrate de potassium.

Tableau VI.

Influence du butyrate de sodium à différentes concentrations sur la respiration de *Tetrahymena geleii*.

Concentration	A	B	Accélération brute	Accélération corrigée par rapport au témoin
0,001 M	12,8	17,0	+ 32 %	+ 48 %
0,005 M	15,2	20,4	+ 35 %	+ 51 %
0,01 M	14,9	21,4	+ 43 %	+ 59 %
0,02 M	16,4	22,4	+ 36 %	+ 52 %
0,1 M	15,2	15,0	— 1 %	+ 15 %
—	15,8 T	13,2 T	— 16 %	—

On peut se demander si, dans ces conditions, le butyrate est un substrat de la respiration ou un accélérateur de la respiration de base; dans ce dernier cas, il ne serait pas lui-même oxydé. Sans résoudre définitivement la question, nous indiquons que le quotient respiratoire, qui est de 0,93 en l'absence de butyrate, tombe à 0,75 en sa présence, ce qui est un indice de la participation de l'acide gras aux oxydations cellulaires.

Action de diverses substances organiques autres que le butyrate sur la respiration de *Tetrahymena geleii*.

Le Tableau VII montre l'influence d'une série de substances organiques sur la consommation d'oxygène par *Tetrahymena geleii*.

Si l'on fait abstraction des écarts inférieurs à ± 30 % présentés par les résultats expérimentaux, il ressort des chiffres de ce tableau d'une part que, parmi les nombreuses substances essayées, seuls l'acétate de sodium et le butyrate d'éthyle provoquent, à la concentration de 0,01 M, une accélération notable de la respiration, et d'autre part que la pyrocatéchine provoque une certaine inhibition de cette respiration. Il convient de signaler ici que l'arrêt brusque de la respiration après introduction du linoléate de sodium est dû à la lyse pratiquement instantanée des cellules du cilié en présence de cette substance. Cette lyse fait actuellement l'objet d'une étude particulière dont les premiers résultats, communiqués à la Réunion de l'Association Française pour l'Avancement des Sciences en septembre dernier, sont en cours de publication (2).

Tableau VII.

Influence de différents substrats à la concentration de 0,01 M sur la respiration de *Tetrahymena geleii.*

A = Q_{O_2} calculé pour l'heure précédant l'introduction du substrat.
B = Q_{O_2} calculé pour la deuxième heure suivant l'introduction du substrat dans les essais réels.

Les valeurs correspondant aux essais témoins ne recevant aucun substrat d'un bout à l'autre de l'expérience sont désignés par la lettre T.

N° de l'expérience	Substrat	A	B	Accélération brute	Accélération corrigée par rapport au témoin
460304	Glucose	11,0	10,6	— 4 %	+ 13 %
		12,7 T	10,6 T	— 17 %	
460225	Formiate Na	14,7	15,9	+ 8 %	+ 7 %
		14,5 T	14,7 T	+ 1 %	
460715	Ethanol	13,9	16,0	+ 15 %	+ 22 %
		15,1 T	14,1 T	— 7 %	
	Ethanol [1])	13,2	13,6	+ 2 %	+ 4 %
		12,7 T	12,4 T	— 2 %	
460401	Succinate K	11,0	12,7	+ 15 %	+ 8 %
	Malate K	12,3	12,1	— 1 %	— 8 %
	Citrate K	11,6	13,7	+ 18 %	+ 11 %
		11,3 T	12,1 T	+ 7 %	
460318	Inositol	13,5	11,1	— 17 %	— 16 %
	Pyrocatéchine	15,2	6,6	— 43 %	— 42 %
		11,2 T	11,1 T	— 1 %	
460715	Acétate Na	14,4	22,2	+ 55 %	+ 62 %
		15,1 T	14,1 T	— 7 %	
	Acétate Na [1])	13,6	19,1	+ 41 %	+ 40 %
		14,8 T	15,0 T	+ 1 %	
460318	Propionate Na	14,7	17,8	+ 21 %	+ 21 %
		15,2	17,8	+ 18 %	+ 18 %
		11,2 T	11,2 T	0 %	
460401	Isobutyrate K	10,8	15,0	+ 39 %	+ 32 %
		11,3 T	12,1 T	+ 7 %	
460527	Butyrate d'éthyle	8,7	17,6	+ 102 %	+ 87 %
		10,4 T	12,0 T	+ 15 %	
460325	Caproate Na	15,7	12,7	— 19 %	— 7 %
	Caprylate Na	14,0	15,0	+ 7 %	+ 19 %
	Palmitate Na	14,0	15,7	+ 12 %	+ 24 %
	Stéarate Na	11,1	10,8	— 3 %	+ 9 %
	Linoléate Na	11,9	0 (inhibition et lyse)		
		13,2 T	11,7 T	— 12 %	

[1]) Ces essais ont été réalisés avec des ciliés récoltés sur le milieu à l'eau peptonée additionné du substrat (0,01 M) dont on voulait ultérieurement étudier l'influence sur la respiration.

N° de l'expérience	Substrat	A	B	Accélération brute	Accélération corrigée par rapport au témoin
460425	Alcool butylique	16,1	11,1	— 31 %	— 10 %
	α-aminobutyrate Na	12,6	11,3	— 10 %	+ 11 %
	dl-homocystéine	10,5	11,7 [1]	+ 11 %	+ 32 %
	dl-méthionine	10,9	10,8	— 1 %	+ 20 %
		15,3 T	12,0 T	— 21 %	
460506	Acéto-acétate Na - α-hydroxybutyrate Na	12,4	10,1	— 19 %	+ 8 %
		12,7	10,3	— 19 %	+ 8 %
	Crotonate Na	13,0	10,0	— 26 %	+ 1 %
		12,3 T	9,0 T	— 27 %	

[1]) Compte tenu d'une légère correction pour l'autoxydation de la substance au cours d'expérience.

Discussion des résultats.

L'ensemble des résultats précédents appelle les remarques suivantes:

1°. L'accélération de la respiration en présence des molécules organiques, même de celles qui paraissent avoir l'action la plus marquée, est faible par rapport à celle que l'on observe avec la levure de boulangerie, par exemple. Il est vrai que dans les expériences faites avec ce dernier organisme, les concentrations des substances introduites sont généralement plus élevées que dans les expériences du présent travail (11). Mais ces différences de concentrations ne suffisent pas à expliquer l'écart en question.

2°. La comparaison des résultats actuels et de ceux obtenus par VON DACH (3) dans son travail sur le flagellé *Astasia Klebsii* montre des analogies intéressantes: aucune accélération de la consommation d'oxygène en présence de glucose ou d'autres sucres, non plus qu'en présence de fumarate, succinate, malonate, citrate, lactate, etc.; accélération importante au contraire en présence d'acétate, propionate et butyrate, de même qu'en présence d'alcool éthylique; la concentration de toutes ces substances étant de 0,05 M.

3°. Le fait que dans nos expériences le glucose (comme d'autres sucres d'ailleurs, non mentionnés dans les tableaux précédents: mannose, galactose, lactose, maltose, à la concentration de 0,03 M), n'accélère par la consommation d'oxygène de *Tetrahymena geleii*,

n'est nullement en contradiction avec les résultats obtenus par Lwoff (8), par Loefer (7) et par Kidder (6) sur l'utilisation du sucre par le cilié. L'action du microorganisme sur la glucose et sur quelques autres sucres, lévulose, galactose, maltose, se manifeste par la formation d'acide. Nous pouvons confirmer cette formation d'acide. Nos résultats actuels indiquent simplement que l'utilisation du glucose, même en aérobiose, n'est pas accompagnée de l'oxydation soit du glucose lui-même, soit de l'un de ses produits de scission.

Résumé.

Tetrahymena geleii provenant de cultures âgées de 70 heures, étudié à l'état non proliférant en milieu minéral tamponné à pH 7.3, à la température de 22° C., présente une respiration de base correspondant à Q_{O_2} voisin de 14. La consommation d'oxygène de cet organisme est accrue de 54 % en présence de butyrate de sodium 0,01 M; elle est également accrue en présence d'acétate de sodium et de butyrate d'éthyle. Elle n'est pratiquement pas modifiée ni par le glucose ni par les acides dicarboxyliques participant au cycle de Krebs. Elle est inhibée de 50 % par la pyrocatéchine 0,01 M.

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(Hopkins Marine Station of Stanford University, Pacific Grove, California).

STUDIES ON THE PIGMENTS OF THE PURPLE BACTERIA

III. The yellow and red pigments of *Rhodopseudomonas spheroides*

by

C. B. VAN NIEL

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1. Certain strains of non-sulfur purple bacteria exhibit a remarkable color change from yellowish-brown to deep red when cultures grown in the absence of air are subsequently exposed to oxygen. The process responsible for this change is not a rapid one; usually it requires several hours to go to completion (1). Since a variety of yellow and red carotenoids have been isolated from the group of purple bacteria [1]) it appeared possible that the pigments involved in the color change might belong to this group.

It is true that FRENCH has concluded from the absorption spectra of extracts of brown and red cultures of such strains that the red color would be due to a pigment which is not a carotenoid (2). But this conclusion was based on a computed absorption curve for the red pigment obtained by subtraction of the absorption spectrum of a brown from that of a red extract, and it has been pointed out [(1), p. 70] that the above interpretation would be invalid if the yellow components were transformed in the course of the reactions causing the color change.

An attempt to study the phenomenon had to start with a characterization of the pigment system of the corresponding organisms. This in itself is still a fruitful field for investigation because so little is definitely known concerning the nature of the pigments and their distribution among different purple bacteria. It was made possible through the generosity of Prof. L. ZECHMEISTER, in whose laboratory and under whose able guidance the work was started.

[1]) This subject has been recently reviewed [(1), p. 53–70]; for this reason specific references are here omitted.

2. There was at my disposal a considerable quantity of dried bacteria, grown from pure cultures of *Rhodopseudomonas spheroides* in yeast extract-malate media [1]) under semi-anaerobic conditions in continuous light. The cells were separated from the medium by means of a Sharpless Supercentrifuge, dried in shallow layers on glass plates at 40° C. in air, and stored in the dark under nitrogen since 1935-'36. This material consisted of two batches, representing two strains of *Rh. spheroides*; one had been isolated from mud in Pacific Grove, California (No. 28), the other in Delft, Holland (No. 30). Both were used separately for the extraction and separation of the major carotenoid components.

After finely grinding the dry flakes in a pebble mill the powder was exhaustively extracted with a methanol-petroleum ether mixture. The solution was filtered, and the pigments transferred to the petroleum ether phase by the addition of water. The pigment solution, after washing and drying, was analyzed chromatographically on a calcium carbonate column, development being effected with petroleum ether.

Five zones were obtained in this manner [2]):

I. A bluegreen top layer, containing the (decomposed) bacteriochlorophyll.

II. A 15 mm wide band, composed of at least six small fractions of purplish, red, and yellow adsorbates.

III. A 19 mm wide band of magenta color, well separated from the others, and composed of a dark upper and a lighter bottom zone.

IV. An 18 mm wide band of very intense yellow color, equally well separated.

V. A narrow, 3 mm wide band of a pale yellow adsorbate.

The bulk of the pigment was contained in the 3rd and 4th zones; these were cut out, the pigments eluted with ethanol, transferred to petroleum ether, and the solutions rechromatographed until homogeneous. While for the purification of the yellow pigment calcium carbonate columns and development with petroleum ether proved satisfactory, a resolution of the magenta adsorbate by the same treatment was too slow. Addition of acetone to the petroleum

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[1]) Composition: H_2O, 1000; $(NH_4)_2SO_4$, 1 g; $MgCl_2$, 0.5 g; K_2HPO_4, 3 g; Na-malate, 3 g, yeast autolysate, 1 ml; adjusted to pH 6.8–7.0.

[2]) The specific data pertain to a column of 60 mm diameter, and a pigment extract from 50 grams of dry bacteria, containing about 100–200 mg carotenoids.

ether, even as little as 0.5 %, caused a very fast migration of the adsorbed zone, accompanied by discoloration, and did not constitute an improvement. Better results were obtained by using calcium hydroxide („Shell brand") as adsorbent, and developing with petroleum ether containing 2.5 % acetone. Invariably the red solutions resulting from the elution of the corresponding bands on the first columns appeared on rechromatographing to consist of some 10 constituents; most of these turned out to be stereoisomers of the main pigment, as shown by the absorption spectra of their solutions after iodine-catalyzed isomerization according to the procedures developed by ZECHMEISTER (3).

The main yellow and red pigments, both all-*trans* compounds, were crystallized from benzene solutions by the addition of methanol. The final crystalline samples used for the characterization of these substances represented products that had been recrystallized at least three times.

From the 5th, pale yellow zone a petroleum ether solution was prepared for determination of the principal absorption bands; the amount of pigment was insufficient to secure crystals for more complete characterization.

As evidenced by the complex second adsorption band the original pigment extract contained a variety of additional carotenoids in small amounts. Several of these were purified by repeated adsorption, and were found to be stereoisomers of the major yellow and red pigments by measurements of the absorption maxima before and after iodine-catalyzed isomerization (3). Particularly the red pigment appears liable to give rise to isomers; in this respect it behaves not unlike the principal carotenoid pigment of *Rhodospirillum rubrum*, spirilloxanthin (4).

Since it was conceivable that the profusion of stereoisomers was due to the preliminary drying of the cells, or to the long storage period of this material, or that they had been formed during the rather lengthy extraction and purification process, the results obtained with the dehydrated bacteria were checked with freshly grown cultures. In this investigation the cells from 20 25 liter cultures were collected by centrifugation and extracted directly with acetone and methanol. The pigments were again transferred to petroleum ether, and this solution subjected to a chromatographic analysis. It was thus ascertained that in fresh cells of *Rhodopseudomonas spheroides* the deep yellow and the red pigments in their all-*trans* configuration comprize at least 95 % of the total

carotenoid content, and that stereoisomers are virtually absent.

Comparison of the composition of the pigment systems of the dried cells representing the two different strains of *Rh. spheroides* showed complete agreement, as did also later experiments with fresh cultures of these and of three additional strains of the same species. Identity of the two main pigments was established by comparison of the absorption spectra, by mixed chromatograms, and mixed melting point determinations. It seems therefore probable that the pigment system of other strains of *Rh. spheroides* can also be characterized as composed of bacteriochlorophyll and only two major carotenoids. The properties of the latter are described in more detail in the following section.

3. The red constituent, crystallized from benzene-methanol solutions, forms dark red rectangular leaflets, reminiscent of SCHÖN's gazaniaxanthin (5,6), with a M.P. of 148° C. (corr.). Its absorption maxima in different solvents, as determined with a Zeiss Evaluating Grating Spectroscope, are listed below:

Solvent:	Absorption maxima, mμ:		
Carbon bisulfide	557	519.6	486.4
Benzene	536	499	464.5
Chloroform.	(531.5?)	493	(462?) not sharp
Petroleum ether (B.P. 60–70° C.)	516.5	487.2	455

Its extinction curve in benzene solution, computed as $E^{1\%}_{1cm}$ values from the transmission measurements made with a Beckman spectrophotometer, is presented by the dotted line in Fig. 1; determinations were made at 5 mμ intervals except in the vicinity of the maxima and minima where readings were taken 1 or 2 mμ apart.

The maximum at 382 mμ represents a small „*cis*-peak" (3), as shown by extinction curves of the isomerized pigment in benzene solution which exhibit this peak in a greatly accentuated form. The presence of *cis*-isomers at the same time causes a depression of the maxima at longer wavelengths. Consequently the extinction coefficients of the all-*trans* carotenoid are probably slightly higher than those represented in the graph. However, the difference cannot be greater than 2 %.

The pigment is strictly epiphasic in petroleum ether — 85 % methanol, and less than 0.1 % is extracted from petroleum ether by 90 % methanol.

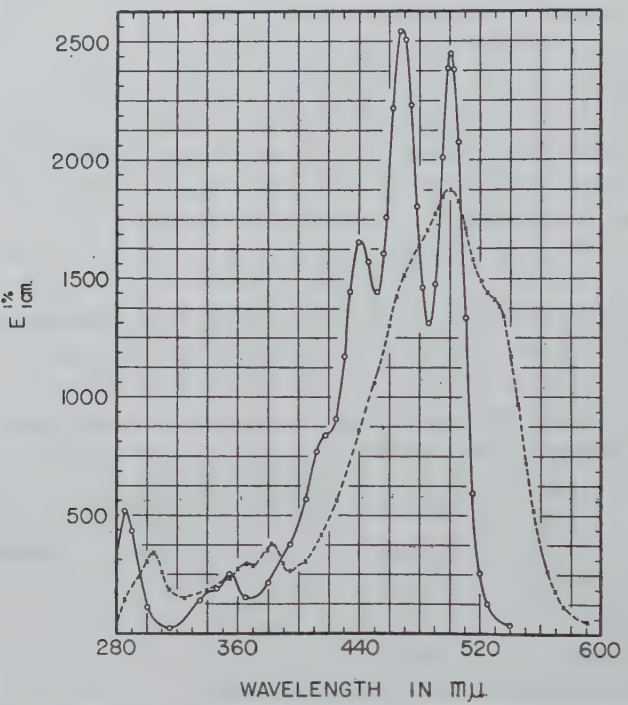

Fig. 1. Extinction curves $\left(E^{1\,\%}_{1\,cm}\right)$ of the yellow (solid line) and red (dotted line) components of the carotenoid pigments of *Rhodopseudomonas spheroides* in benzene solution.

Rather typical is its behavior when adsorbed on calcium carbonate; it forms a brownish-purple zone which migrates extremely slowly when developed with petroleum ether, and is rapidly discolored to a brownish-orange, very narrow band which is washed through the column at a great rate if the petroleum ether contains a trace (less than 0.5 %) of acetone.

The absorption spectrum is very different from that of gazaniaxanthin, but rather closely resembles that of celaxanthin (7) as far as the maxima in carbon bisulfide, benzene, and petroleum ether are concerned. However, the much higher M.P. of celaxanthin (209° C.) argues against identity. Also the carotenoid

which KARRER and SOLMSSEN (8) have obtained from purple bacteria cultures and named rhodovibrin has similarly situated absorption maxima in carbon bisulfide (556 and 517 mμ). Especially in view of the source of rhodovibrin it might tentatively be considered the same as the red component from *Rh. spheroides.* On the other hand, the statement that rhodovibrin resembles rhodopin in crystal form and M.P. (168° C.), though not in the position of the absorption maxima, render this doubtful, and rather suggest that rhodovibrin would be a stereoisomer of rhodopin.

The intensely yellow component crystallizes in the form of needles with a M.P. of 132° C. (corr.); its absorption maxima in various solvents are as follows:

Solvent:	Absorption maxima, mμ:		
Carbon bisulfide	521.5	487.1	455.4 (426)
Benzene	499.7	466.6	438
Chloroform.	497	464	(436)
Ethanol	486.6	454	(426)
Hexane	486.4	454	(426)
Petroleum ether (B.P. 60–70° C.)	486	454	426.5

Fig. 1 also presents the extinction curve of this pigment in benzene solution, determined as for the red carotenoid (solid line). Here, too, the maximum at 355 mμ is caused by the admixture of small amounts of *cis*-isomers, so that the true extinction coefficients for the all-*trans* isomer are actually slightly larger.

Like the red pigment, it is epiphasic towards 85 % methanol; 90 % methanol extracts a trace, and 95 % methanol some (about 2 %) pigment from petroleum ether.

There is some resemblance between the absorption maxima of this substance and of β-carotene, but again identity is improbable on account of the large difference in melting points (183° C. for β-carotene). It is conceivable that this is the pigment which KARRER and SOLMSSEN (8) observed and referred to as possibly consisting of β-carotene.

The very small fraction of faint-yellow pigment mentioned above showed absorption bands in petroleum ether at 470 and 442.2 mμ; it may well be identical with flavorhodin (8, 9).

4. From the superimposed extinction curves it will be seen that

the determination of the extinction values at 530 and 500 mμ of a benzene solution containing both pigments permits the computation of the amounts of each component. The equation used is developed as follows:

For 1 mg of red pigment in 250 ml benzene

$$E_{530} = 0.553;\ E_{500} = 0.742.$$

For 1 mg of yellow pigment in 250 ml benzene

$$E_{530} = 0.0294;\ E_{500} = 0.97474.$$

In a solution containing per 250 ml y mg yellow and r mg red pigment the relation holds that:

$$E_{530} = E_{530}^{\text{Yell.}} + E_{530}^{\text{Red}},\ \text{and}\ E_{500} = E_{500}^{\text{Yell.}} + E_{500}^{\text{Red.}};$$

consequently:

$$E_{530} = 0.0294\ y + 0.553\ r;\ E_{500} = 0.97474\ y + 0.724\ r,$$

whence $y = 1.026\ E_{500} - 0.76122\ r$, and

$$r = 1.8845\ E_{530} - 0.05684\ E_{500}.$$

Because other carotenoids are not present in significant amounts, it was thus possible to determine quantitatively the red and yellow pigments in cultures of *Rh. spheroides* without resorting to a quantitative separation procedure.

Preliminary investigations with liquid cultures showed that a complete extraction of the pigments can best be achieved in the following way. First the bacteria are concentrated to a dense suspension by centrifugation; to this are added 7 volumes of acetone and 2 volumes of methanol while stirring. The bacteria flocculate so that they can be readily filtered off on a small cotton plug, conveniently placed at the upper end of the stem of a funnel; the residue is here repeatedly extracted and washed with small amounts of methanol, acetone, and benzene until colorless. The filtrate, upon addition of H_2O, separates into two phases, the benzene layer containing all the carotenoids and most of the bacteriochlorophyll. It is washed free of acetone and methanol with H_2O, dried with Na_2SO_4, and diluted with benzene to the desired pigment concentration, whereupon the total volume is measured and the extinction at 500 and 530 mμ determined.

Special experiments showed that it is unnecessary to saponify the solution in order to eliminate bacteriochlorophyll. Nevertheless, saponification of the original extract may sometimes be useful in that it causes a faster separation of the benzene phase and

tends to avoid emulsions; for this reason it was often included.

Twenty-five to 50 ml of a full-grown culture are sufficient to prepare 25 ml of the final benzene solution of adequate color intensity for the transmission measurements. Concentration of the bacteria from the liquid culture sample is considerably facilitated by adding $^1/_5$ volume of acetone before centrifugation; the cells settle out rapidly and can easily be redispersed in a small amount of water, while the acetone in so low a concentration does not extract any pigment.

5. Based upon this methodology the relation between the pigments of yellowish-brown cultures of *Rh. spheroides*, grown under anaerobic conditions, and of cultures turned red as a result of exposure to oxygen was investigated in the following manner.

The mineral-malate medium with 0.1 % yeast autolysate was sterilized in glass-stoppered bottles of 1 liter capacity, each containing approximately 900–950 ml of solution. After inoculation the bottles were completely filled with sterile medium, and incubated at 28° C. in continuous light. When the cultures had reached maximum development — in 1 to 4 days, depending upon the amount of inoculum used — they were thoroughly mixed and the contents dispersed with aseptic precautions into the requisite number of sterile 125 ml bottles and 500 ml flasks. The bottles were again completely filled; the flasks only to a depth of about 1–2 cm. For most experiments one half of the bottles and flasks was further incubated at 28° C. in darkness, the other half while exposed to continuous illumination. In the flask cultures aerobic conditions were maintained by constant agitation on a shaking machine. For analysis of the pigment content 25 to 50 ml samples were removed and treated as above outlined at a time when the color change of the aerobic cultures had become apparent, usually after a lapse of 12–16 hours (incubation overnight), while occasionally the incubation period was extended to 40 hours. The table below presents the results obtained in two independent experiments with strain No. 28.

It will be noticed that the total amount of pigment per 50 ml of the anaerobic cultures in the light exceeded by some 20 μg that of the dark anaerobic cultures. Similar differences were frequently observed in experiments for which the mother culture, lightly inoculated, was incubated for not more than 4 days. Both the yellow and the red component apparently contributed to the

Table I.

Pigment composition of cultures of *Rhodopseudomonas spheroides* in the absence and presence of oxygen, incubated in darkness and in light. Quantities represent μg pigment per 50 ml culture.

Incubation period	Pigment Component	Anaerobic		Aerobic		Increase	
		Dark	Light	Dark	Light	Dark	Light
16 Hrs.	Yellow	189	201	152	97	— 49	— 104
	Red	97.5	103	155	202	+ 52	+ 99
	Total	286.5	304	307	299	+ 3	— 5
40 Hrs.	Yellow	186	195	146	84	— 49	— 111
	Red	101.5	112	163	220	+ 51	+ 108
	Total	287.5	307	309	304	+ 2	— 3

increase. That it actually represents an increase, due to a slight additional growth of the bacteria in the light, and not to a destruction of pigment under anaerobic conditions in darkness, was shown by comparison with pigment determinations on samples taken directly from the mother culture. Such discrepancies can be avoided by incubating the mother cultures for longer periods of time, but this generally causes a perceptible reduction in the rate of color change. In anaerobic cultures the ratio of yellow to red component is frequently around 2 : 1, although occasionally ratios as high as 10 : 1 have been observed.

The color change induced by incubation under aerobic conditions is obviously due to a shift in this ratio; it may, under favorable circumstances, reach a value of 1 : 3. And the shift results from the combined effect of a disappearance of the yellow pigment, accompanied by an increase in the red one. The total amount of pigment remains rigorously constant within the limits of accuracy of the analytical procedure. The quantitative relationships between the changes of the two pigment components suggest a conversion whereby a unit weight of yellow pigment is transformed into an equal quantity of the red carotenoid.

Comparison of the results obtained with aerobic cultures in darkness and in light reveals that for the same time interval the transformation is considerably greater in illuminated suspensions. So far, it is not possible to conclude whether this is due to a direct photochemical effect on the conversion reaction or to an indirect effect of illumination on the viability and general physiological activity of the bacteria. In order to appreciate this distinction it is necessary

to take cognizance of the fact that the conversion of the yellow to the red component strictly depends upon the presence of viable bacteria.

FRENCH (2) has previously shown that aqueous extracts of the brown form of *Rh. spheroides*, obtained by grinding the cells in a hypodermic syringe, contain the entire pigment system of the organisms in a water-soluble form, presumably as protein-pigment complexes, and that such solutions remain unaltered in color when exposed to oxygen for a period of days. From these observations he concluded: „It therefore seems likely that the brown to red change is an enzymatic process taking place only in the intact bacteria and possibly having some relation to cell respiration".

In support of this conclusion the following evidence is submitted. If the mother culture is heated for 5 minutes at 90° C., the subsequent color change upon exposure to oxygen is prevented; likewise suspensions of brown cultures of *Rh. spheroides* do not turn red when shaken in air in the presence of toluene. Under these conditions the composition of the carotenoids in the cells undergoes no modification, thus demonstrating that the transformation is closely associated with the physiological activities of the organisms.

Finally it should be mentioned that the various results relating to the color change have been observed with four different strains of *Rh. spheroides*. It seems probable that the analogous color change exhibited by cultures of *Rh. capsulatus* is due to a similar mechanism especially because the yellow and red carotenoids isolated from *Rh. spheroides* cultures have also been found in the cells of *Rh. capsulatus*. However, some preliminary studies on the pigment system of the latter species have indicated that it is composed of a more complex mixture of carotenoids than that of *Rh. spheroides*. And it is obviously impossible to carry out quantitative studies until the individual components have been characterized.

6. Present evidence indicates that the color change is not reversible. FRENCH, from his experience, stated: „. . . . nor has the red form been changed back to brown" (2). This has been confirmed by my own investigations, which were carried out with mother cultures prepared in well-aerated flasks, and therefore deep red in color. Such cultures failed to turn brown when subsequently incubated anaerobically, even in the presence of substrates like glucose, fumarate, and mixtures of these substances which might permit active metabolism in the absence of air.

In a single instance a decrease in red pigment amounting to 110 μg per 100 ml was observed, accompanied by an increase in the yellow component of 89 μg. However, this culture was found to be contaminated; with pure cultures such changes have not been obtained. Analysis of the pigments of suspensions incubated anaerobically in the light, especially when supplied with additional substrates, frequently did demonstrate an increase in the quantity of yellow pigment, but this was invariably linked with an increase in total pigment content, and not with a corresponding decrease of the red constituent. Consequently it is probable that these results stem from the new-formation of carotenoids due to continued growth of the bacteria. Since this growth occurred in the absence of oxygen the pigment production should logically be expected to affect principally the yellow component. It seems justified to conclude that the red carotenoid does not function in the metabolism of *Rh. spheroides* as an hydrogen acceptor.

As long as the chemical constitution of the pigments has not been ascertained it seems best to refrain from formulating hypotheses concerning the detailed mechanism of the conversion, and from speculating on the significance of the phenomenon for the problem of the function of carotenoids in metabolism.

It is a pleasure to express my gratitude to Professor L. Zechmeister for his aid and advice, and to Mrs. M. North Allen for assistance in part of the experimental work.

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(Division of Plant Nutrition, University of California, Berkeley).

CLOSTRIDIUM KLUYVERI

by

H. A. BARKER

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In July, 1935, I arrived at the Laboratorium voor Mikrobiologie in Delft eager to try to obtain some experimental support for the theory, developed by my former teacher, Professor C. B. VAN NIEL, that methane is formed in methane fermentations of organic compounds by a reduction of carbon dioxide. While discussing this problem with Professor KLUYVER, he suggested that a most promising line of attack would be to study the decomposition of ethyl alcohol by enrichment cultures of methane-producing bacteria. This I undertook to do.

Enrichment cultures were started by adding alcohol, calcium carbonate and the usual inorganic salts to tapwater, inoculating generously with canal mud and incubating the mixture anaerobically (1, 2). After a few days a vigorous gas production commenced and chemical analysis showed that the alcohol was being oxidized to acetic acid while the carbon dioxide which might have been expected from the neutralisation of the acid by the carbonate disappeared and was replaced by an equivalent amount of methane (Equation 1).

$$2\,CH_3CH_2OH + CaCO_3 \quad (CH_3COO)_2Ca + CH_4 \qquad (1)$$

Not every enrichment culture gave this result (3). In almost half the cultures alcohol was not oxidized entirely to acetic acid, but a considerable part was converted into some higher fatty acid which could be easily detected by its odor and by Duclaux distillation. In order to identify the higher acid or acids, a large culture was prepared and after the fermentation was complete the medium was acidified and steam distilled. To my surprise the first part of the distillate contained a considerable quantity of an acid which floated as an oily liquid on top of the water. The low solubility indicated it must contain more than four carbon atoms. Since I

was not aware of any previous observations of the microbial formation of a fatty acid of this sort in high yield [1]) particularly from so simple a substrate as ethyl alcohol, I immediately realized that I had made a discovery of considerable interest. The first thing I did after completing the distillation was to show a sample of the oily liquid to Professor KLUYVER, and I recall that we spent a considerable time discussing the probable identity of the acid and the reactions by which it might be formed.

Subsequently it was shown that the water-insoluble acid is n-caproic acid. Acetic, butyric and caproic acids account for nearly all the ethyl alcohol decomposed in enrichment cultures. A considerable quantity of carbon dioxide is used up and an equivalent amount of methane is formed.

From the microbiological point of view it was important to find out whether all the observed chemical transformations, namely the conversion of ethyl alcohol to acetic, butyric and caproic acids and of carbon dioxide to methane, are due to a single organism or to several different organisms. The early work was done entirely with crude enrichment cultures containing a great variety of bacteria and therefore it was not possible to arrive at any certain conclusions on this point. However, microscopic examination of numerous cultures revealed that two types of bacteria are always abundant when butyric and caproic acids are formed. One is *Methanobacterium omelianskii*, the organism that causes the anaerobic oxidation of ethyl alcohol to acetic acid as shown in equation 1. The other is a large, generally Gram-negative, motile spore-forming rod. This latter organism appeared to be directly responsible for the formation of caproic and butyric acids since there was a close correlation between its presence and their formation. The large spore-former never occurred alone in these cultures but was always accompanied by the methane-producing bacterium.

These observations gave rise to the idea that butyric and caproic acids are produced by the synergistic action of these two bacteria. The methane-producing organism was assumed to cause the oxidation of ethyl alcohol to acetaldehyde which was then condensed to the C_4 and C_6 acids under the influence of the large

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[1]) Later on a search of the literature revealed that BÉCHAMP, a student of PASTEUR, had in 1868 observed the conversion of ethyl alcohol to butyric and caproic acids (4).

spore-former (3). This explanation is in accordance with the classical but poorly supported acetaldol theory of butyric acid formation. It is interesting to note that the most obvious alternative explanation, namely that acetic acid rather than acetaldehyde is the essential compound formed by the methane bacterium and used by the spore-former, was not considered seriously at that time.

Before any further progress could be made in understanding the caproic acid fermentation, it was obviously necessary to obtain pure cultures of the two bacteria. *Methanobacterium omelianskii* was isolated in June, 1938 (5), and the spore-former, which was given the name *Clostridium Kluyveri*, was isolated in February, 1939 (6).

The original isolation of *Cl. Kluyveri* proved to be rather difficult because its nutritional requirements were not understood. The enrichment medium in which the organism was first observed and later studied was a simple synthetic medium containing ethyl alcohol as the only added organic compound. As soon as pure culture isolation was attempted it became apparent that separate colonies of *Cl. Kluyveri* cannot grow in this same medium. When deep agar shake cultures were inoculated in serial dilution from an enrichment culture, some growth of *Cl. Kluyveri* occurred in the first and second dilutions but in higher dilutions only colonies of *M. omelianskii* and various contaminants appeared. As it seemed possible that this might be due to lack of one or more growth factors supplied in the enrichment medium by associated bacteria, I tried adding a small amount of yeast autolysate. This seemed to have a slightly beneficial effect, so higher concentrations were tried and in this way it was found that isolated colonies of *Cl. Kluyveri* can grow moderately well in a medium containing ethyl alcohol and an abnormally high concentration of yeast autolysate. Whereas most bacteria with fairly complex nutritional requirements grow well with 5 volumes per cent yeast autolysate, *Cl. Kluyveri* shows only a slight response to twice this amount and does much better with 20, 30 or even 40 volumes per cent. By the use of such high concentrations of yeast autolysate it was eventually possible to isolate several pure cultures of *Cl. Kluyveri*, although considerable difficulty was experienced due to the rapid growth of the more common putrefactive clostridia in so rich a medium.

As soon as pure cultures were obtained they were tested for ability to grow and produce caproic acid in various media (6). The results were rather disappointing. In an alcohol-carbonate medium

without yeast autolysate, no growth occurred. When the medium was supplemented with more than 10 volumes per cent yeast autolysate, the organism grew to some extent but the amount of caproic acid formed was always very small in comparison with that obtained in the enrichment cultures.

The experiments of TAHA (6) indicated that the caproic acid is derived mainly from ethyl alcohol, although small amounts of lactate and acetate present in the yeast autolysate also disappeared. A little hydrogen is always formed but no methane or carbon dioxide. The amino acids in the yeast autolysate are not attacked to any appreciable extent judging from the very small formation of ammonia. Furthermore, other amino acid-containing substrates such as peptone and tryptone cannot replace yeast autolysate as an essential nutrient. Neither glucose nor any other common carbohydrate is fermented.

In view of the relative inactivity of *Cl. Kluyveri* in pure culture under the conditions then used, it seemed desirable to find out how it would behave in mixed culture with *M. omelianskii*, *i.e.*, under conditions more closely simulating those in enrichment cultures. The results were rather significant. The following facts were established:

1. In an alcohol-carbonate medium without yeast autolysate only *M. omelianskii* grows and the only fatty acid formed is acetic acid.

2. In an alcohol-carbonate medium supplemented with a little yeast autolysate (1—9 vol., per cent) both organisms grow well and a considerable amount of ethyl alcohol is converted into caproic acid. A small quantity of acetic acid also accumulates.

3. With a high concentration of yeast autolysate (27 vol., per cent) only *Cl. Kluyveri* develops. Much less alcohol is used, but the main product is caproic acid. Later experiments by BORNSTEIN showed the formation of caproic acid is roughly proportional to the concentration of yeast autolysate added.

From these observations it could be concluded that *Cl. Kluyveri* requires some constituent of the yeast autolysate in relatively small amount even in the presence of *M. omelianskii*. This constituent is probably in the nature of a growth factor, since it evidently does not contribute directly to the formation of caproic acid. A second conclusion was that the relatively large amount of yeast autolysate required by pure cultures of *Cl. Kluyveri* supplies some compound other than ethyl alcohol which is directly involved in

the formation of caproic acid. The same or a similar compound is evidently formed by *M. omelianskii* from alcohol.

The next step in the investigation was the fractionation of the yeast autolysate in an effort to identify the constituent needed in relatively large amount. This and many of the experiments to be described subsequently were carried out by B. T. BORNSTEIN (7). Since there were already several indications that acetic acid might be involved in caproic acid formation, the yeast autolysate was first acidified and steam distilled and both the distillate and residue were tested for growth stimulating activity. It was immediately observed that the volatile acid fraction greatly improved the growth in a basal medium containing alcohol and a moderate amount of yeast autolysate, while the residue from the steam distillation was completely inactive. The volatile acid fraction was shown to consist mainly of acetic acid and the growth stimulating activity of the fraction could be entirely accounted for by the acetate it contained. The addition of larger amounts of acetate to the basal medium improved the growth far beyond the highest level that had previously been attained with maximal concentrations of yeast autolysate.

Even though most of the yeast autolysate could be replaced by acetate, a small amount was still found to be essential for growth in the original basal medium. With an excess of alcohol and acetate, growth increased with yeast autolysate concentration up to about 0.5 vol. per cent. However, later experiments showed that this small amount can be replaced completely by two growth factors, biotin and para-aminobenzoic acid. It is therefore now possible to grow *Cl. Kluyveri* successfully in a synthetic medium containing ethyl alcohol, sodium acetate, biotin, para-aminobenzoic acid, phosphate buffer and the usual inorganic salts. When an active inoculum is used, good growth can be obtained in 24 hours at 37° C.

Cl. Kluyveri is very restricted with respect to the substrates it can use. It has already been mentioned that sugars and amino acids are not attacked to any appreciable extent. The same applies to lactate and pyruvate. All attempts to replace ethyl alcohol by other alcohols have so far been unsuccessful. However, acetate can be replaced as a partial growth substrate by two other fatty acids, propionate and butyrate. Higher fatty acids are used very poorly if at all by the strain that has been most extensively studied so far.

After having established the nutritional requirements of *Cl.*

Kluyveri, its catabolic reactions were investigated by BORNSTEIN by the analysis of growing cultures. He found that the products formed from ethyl alcohol and acetate are butyrate, caproate and hydrogen. No carbon dioxide is produced; in fact there appears to be a very small disappearance of carbon dioxide from the medium.

The quantity of hydrogen formed is relatively small, being of the order of 13 mM per 100 mM of alcohol decomposed. The relative amounts of butyric and caproic acids are determined by the relative concentrations of alcohol and acetate in the medium. If acetate is present in excess, butyric acid is the most abundant product. The main reaction can then be expressed by the equation

$$CH_3CH_2OH + CH_3COOH \rightarrow CH_3CH_2CH_2COOH + H_2O. \quad (2)$$

If on the contrary alcohol is present in excess, caproic acid is the main product and the overall equation for the reaction is

$$2\,CH_3CH_2OH + CH_3COOH \rightarrow CH_3(CH_2)_4COOH + 2\,H_2O. \quad (3)$$

The fact that butyric acid is the main product when a limited amount of alcohol is available while caproic acid predominates when alcohol is in excess indicates that butyric acid is an intermediate in the conversion of acetic into caproic acid, as follows:

$$CH_3COOH \xrightarrow{+C_2H_5OH} CH_3(CH_2)_2COOH \xrightarrow{+C_2H_5OH} CH_3(CH_2)_4COOH. \quad (4)$$

This interpretation is further supported by the observation that when butyric acid is substituted for acetic acid as a partial substrate, caproic acid is formed according to the equation:

$$CH_3CH_2OH + CH_3(CH_2)_2COOH \rightarrow CH_3(CH_2)_4COOH + H_2O. \quad (5)$$

Shortly after these facts were uncovered in the spring of 1944 the opportunity presented itself of further analyzing the reactions involved in butyric and caproic acid synthesis by using the long-lived radioactive carbon isotope, C^{14}. It was especially fortunate that just at this time Dr M. D. KAMEN, one of the codiscoverers of C^{14} (8), was relieved of his wartime duties and was able to give invaluable assistance in carrying out the isotope experiments (9).

The first experiments were designed primarily to find out what parts of the butyric and caproic acid molecules are derived from acetic acid. For this purpose carboxyl-labeled acetic acid was prepared from isotopic carbon dioxide and methyl bromide by the

Grignard reaction. The labeled acetic and a smaller amount of unlabeled ethyl alcohol were then fermented by *Cl. Kluyveri* and the reaction products and unfermented substrates were separated and examined for the presence of C^{14} with the aid of a Geiger counter. The following facts were established:

1. Both butyric and caproic acids contain the isotope originally added in the carboxyl group of acetic acid. In butyric acid, C^{14} is present in both the carboxyl and beta carbon atoms, approximately 55 per cent of the total being present in the former and 45 per cent in the latter position. The alpha and gamma carbon atoms do not contain a significant quantity of the isotope. In caproic acid, roughly one third of the C^{14} is in the carboxyl group. The remainder is probably in the beta and delta positions, though this has not yet been proven.

2. Only a relatively insignificant quantity of the isotope is present in the residual ethyl alcohol.

3. No active carbon dioxide is formed.

4. The isotope content of the residual acetic acid is lower than that of the initial acetic acid by the amount to be expected if the alcohol is oxidized to acetic acid.

These results are consistent with the view that ethyl alcohol is oxidized to acetic acid or some reactive derivative such as acetyl phosphate (reaction 6) which is in approximate isotopic equilibrium with acetic acid (reaction 7). The slightly unequal distribution of the isotope in the carboxyl and beta carbon atoms of butyric acid indicates that complete equilibrium is not attained. A condensation then occurs between the reactive derivative and acetic acid to form acetoacetic acid or some related compound which is reduced to butyric acid (reaction 8). The postulated reactions may be represented by the following equations based upon LIPMANN's acetyl phosphate theory (10):

$$CH_3CH_2OH + K_2HPO_4 \rightarrow CH_3COOPO_3K_2 + 4\,H \qquad (6)$$

$$CH_3^{*}COOPO_3K_2 + ADP \rightleftarrows CH_3^{*}COOH + ATP \qquad (7)$$

$$CH_3^{*}COOPO_3K_2 + CH_3^{*}COOH + 4\,H \rightarrow CH_3CH_2CH_2^{*}COOH^{*} + K_2HPO_4 + H_2O. \qquad (8)$$

Caproic acid labeled in the carboxyl, beta and delta positions is presumably formed by a further condensation of the labeled butyric acid, analogous to reaction 8. This condensation will be discussed in more detail further on.

A possible alternative explanation for the dilution of the labeled acetic acid and other observations is that the alcohol and acetic acid are reversibly oxidized and reduced, respectively, to acetaldehyde which then condenses to butyric acid. This explanation seems to be excluded by two facts. One is the unequal distribution of the isotope in butyric acid. If butyric acid were formed by condensation of two molecules of acetaldehyde, the distribution of the isotope would have to be equal. Only by a condensation of two dissimilar molecules can one account for an unequal distribution. The other pertinent fact is the practical absence of the isotope from the residual alcohol. It is known that ethyl alcohol and acetaldehyde are components of a readily reversible redox system. If acetaldehyde were formed from acetic acid, C^{14} should get into the ethyl alcohol in large amount. This does not occur.

The role of acetic acid in the catabolism of *Cl. Kluyveri* is now considerably clearer. Although we do not yet know in any detail the mechanism of the conversion of ethyl alcohol and acetic acid into butyric acid, it is certain that the process is not a simple condensation with a loss of water, but involves coupled oxidation-reduction reactions. The alcohol is evidently oxidized to acetic acid with the removal of four equivalents of hydrogen. A small part of this hydrogen is removed as gaseous hydrogen, but most of it is used to reduce an organic hydrogen acceptor to butyric acid, at least during the first part of the fermentation. This acceptor, which is ultimately able to react with four equivalents of hydrogen is formed from 2 molecules of acetic acid. Since only one molecule of acetic acid is formed by oxidation of alcohol a second molecule must be supplied by the medium. In the latter part of the fermentation butyric acid partially replaces acetic acid to form a hydrogen acceptor which is reduced to caproic acid. From this line of reasoning it is evident that acetic acid and sometimes butyric acid act indirectly as oxidizing agents in butyric acid type fermentations. In this connection it is of interest that *Cl. Kluyveri* is not the only butyric acid bacterium that requires acetic acid as a partial substrate. Dr J. V. Bhat (11) has recently found in this laboratory that the anaerobic decomposition of lactic acid by a *Clostridium* species is similarly dependent upon an outside supply of acetate. This observation probably explains why since the time of Pasteur so little success has been reported in the study of the butyric acid fermentation of lactate.

Now let us return to a consideration of the formation of caproic

acid from butyric acid and a C_2 compound. This can obviously occur in at least two ways. Either the carboxyl group of the C_2 compound (acetylphosphate) can react with the terminal methyl group of butyric acid or the carbonyl group of the latter can react with the methyl group of the former. It is possible to distinguish between these two types of condensation by carrying out a fermentation of ordinary ethyl alcohol and synthetic butyric acid labeled in the carboxyl group with C^{14}. The position of the isotope in the resulting caproic acid must depend on the way in which the condensation occurs. This is illustrated in equations 9 and 10 where the inactive acetyl phosphate and acetic acid are assumed to originate from the oxidation of ethyl alcohol and the butyrylphosphate is assumed to be formed by a transfer of the phosphate group from acetylphosphate to butyric acid, a reaction demonstrated with an enzyme preparation of *Cl. butylicum* by KOEPSELL, JOHNSON and MEEK (12). It can be seen that a condensation of acetylphosphate with labeled butyric acid would yield carboxyl-labeled caproic acid (equation 9) while a condensation of labeled butyrylphosphate or a related compound with ordinary acetic acid would yield caproic acid labeled in the beta position (equation 10).

$$CH_3COOPO_3K_2 + CH_3(CH_2)_2\overset{*}{C}OOH + 4H \rightarrow CH_3(CH_2)_4\overset{*}{C}OOH + K_2HPO_4 + H_2O \quad (9)$$

$$CH_3(CH_2)_2\overset{*}{C}OOPO_3K_2 + CH_3COOH + 4H \rightarrow CH_3(CH_2)_2\overset{*}{C}H_2CH_2COOH + K_2HPO_4 + H_2O \quad (10)$$

The results of this experiment were quite conclusive in showing that caproic acid is formed according to equation 10. Only an insignificant amount of C^{14} is present in the carboxyl group of the caproic acid. A step-wise degradation of this acid by the Barbier-Wieland procedure was carried out by E. R. STADTMAN (13) and it was found that all the isotope is present in the beta position.

It has already been mentionedt hat propionate can replace acetate as a partial substrate for *Cl. Kluyveri*. When a medium containing ethyl alcohol in excess and sodium propionate is fermented, both odd and even number carbon atom fatty acids are formed. The most abundant product is n-valeric acid which is evidently formed according to the overall equation

$$CH_3CH_2OH + CH_3CH_2COOH \rightarrow CH_3(CH_2)_3COOH + H_2O. \quad (11)$$

A further reaction of the valeric acid with a C_2 compound to give n-heptanoic acid occurs only to a very small extent even with an excess of ethyl alcohol.

It is especially significant that the even carbon atom acids, acetic, butyric and caproic, are also formed in good yields in an ethyl alcohol-propionate medium. Acetic and butyric acids cannot be derived from propionate since there is no carbon dioxide production and the very small carbon dioxide utilization cannot account for more than a minute fraction of the observed butyric acid. The formation of these acids therefore constitutes an independent confirmation of the oxidation of ethyl alcohol to acetic acid, a reaction which was postulated on the basis of the earlier isotope experiments.

The added propionate and the intermediately formed acetate evidently compete with each other in the condensation reactions which lead to the synthesis of the 4, 5 and 6 carbon atom fatty acids. Butyric acid can be formed in only one way, by a condensation of two C_2 compounds. Valeric and caproic acids, however, may be formed by two different reactions. Valeric acid may result from both $C_3 — C_2$ and $C_2 — C_3$ condensations, and caproic acid from both $C_4 — C_2$ and $C_3 — C_3$ condensations. A definite conclusion concerning the relative rates of these possible condensation reactions can of course be reached by carbon isotope experiments, but these have not yet been carried out.

In conclusion, I should like to point out the possibility that *Cl. Kluyveri* may have some significance in relation to the problem of petroleum formation. This organism provides an efficient means by which the more ordinary products of the anaerobic decomposition of organic materials, containing 2, 3 and 4 carbon atoms, may be converted into fatty acids containing 4, 5, 6 and 7 carbon atoms. These in turn may be important raw materials for the production of some types of petroleum hydrocarbons.

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(From the Microbiological Laboratory, Princeton University, Princeton, New Jersey).

THE RATES OF GROWTH AND DISINFECTION OF *ESCHERICHIA COLI* IN RELATION TO pH, QUININE AND TEMPERATURE [1])

by

FRANK H. JOHNSON [2]) and ISAAC LEWIN

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The rates of growth and of disinfection of *Escherichia coli* at neutral pH have been studied recently in relation to temperature, hydrostatic pressure and various concentrations of quinine (1). The results indicated that, in a glucose-asparagine-inorganic salt medium, the rate of growth at atmospheric pressure is normally limited by a single enzyme system. According to the evidence presented, the rate of this enzyme reaction increases with rise in temperature in the manner of an ordinary chemical reaction, but at the same time the catalyst undergoes a reversible inactivation through an equilibrium reaction characterized by a high heat and entropy, typical of protein denaturation (2). On the basis of these two reactions involving the same molecule, the relation of growth rate to temperature, from 18° C. to the almost bacteriostatic temperature of 45° C., may be quantitatively described with some accuracy by a theoretical curve, the general equation for which has been derived in accordance with the theory of absolute reaction rates (3, 4, 5). Above 45° C., the cells die at a rate that increases rapidly with rise in temperature, possibly also through a single reaction, with a high apparent activation energy, again suggestive of protein denaturation.

Under increased hydrostatic pressure, or in the presence of quinine, or under certain other conditions, different reactions may limit the net rate of the reproductive process or determine viability, as shown by the complexity of the kinetic data. In both growth

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[2]) Fellow of the John Simon Guggenheim Memorial Foundation, 1945–'46.

and disinfection, however, quinine acts largely as if it promoted the denaturation of one or more essential proteins, in somewhat the manner as that discussed in connection with the effects of quinine and other drugs, such as alcohol, urethane, etc., on bacterial luminescence (6, 7, 8). Thus, the temperature of maximum growth rate, as well as of a given rate of disinfection, is lowered in the presence of quinine. Moreover, hydrostatic pressures up to 500 atmospheres, except at low temperature, were found to oppose fairly strongly the growth-inhibitory and disinfection-promoting actions of the drug. Effects of pressure, similar in magnitude and direction, have been observed with respect to the denaturation of highly purified proteins at increased temperatures (9) and of extracted yeast invertase at alkaline pH (10). The strong effects of pressure are also indicative of reactions involving large molecules, such as proteins.

The present study deals with the influence of pH and quinine on the rates of growth and disinfection, in relation to temperature at normal pressure. An extensive analysis, including the effects of increased hydrostatic pressure, has not been undertaken as yet because of certain complications in the kinetics of the phenomena described.

Methods.

The procedures for cultivating the organisms in a „synthetic" medium, and of counting the viable cells were the same as those employed previously (1). A culture containing between 24,000 and 60,000 cells per cc in the early logarithmic growth phase at 37° C. and pH 6.9 was in all cases used as the source of inoculum for tubes containing the corresponding medium, with or without quinine in a single final concentration of 0.0007 M, and at the desired pH [1]). Two cc of inoculum were added to 10 cc of sterile medium, giving from 4,000 to 10,000 cells per cc at the start of the experiment. Immediately after inoculation these cultures were transferred to one or more constant temperature water baths, and counts of viable

[1]) The pH of the solutions was initially adjusted at room temperature. At a higher or lower temperature there was a slight change in pH, but the difference, as recorded by the Beckman glass electrode, rarely amounted to more than $\pm$ 0.1. For present purposes this margin of error would not seem sufficiently large to justify listing each value of pH as recorded for each experiment, and we will, therefore, refer to the three hydrogen ion concentrations studied in all cases as pH 4.9, 5.9 and 6.9, respectively.

cells were made at intervals over a period of three hours. Aeration was maintained by a gentle stream of air bubbles, rendered aseptic by filtration through sterile cotton. Phosphate buffer was used throughout to keep the pH constant. Although the efficiency of this buffer is much less at a low pH than near neutrality, it was desired to avoid the complications which are sometimes introduced by differences in effects of various buffer systems at a given pH. Moreover, within the range studied, the pH changed only very slightly during the course of the experiment.

Results.

When the pH is suddenly changed from 6.9 to 4.9, whether the temperature is also changed or not, the rate of growth changes practically at once. At 37° C., for example, no apparent increase in number of viable cells takes place during the first hour. Subsequently, however, growth at pH 4.9 is resumed and rapidly attains a rate very nearly the same as that of a control at pH 6.9 (Fig. 1, B). The apparent bacteriostasis possibly results from the killing of some of the cells, more sensitive than others by virtue of a critical stage in cell division or other causes, while a portion of the population continues to reproduce. Where the total change in cell numbers is small, it is difficult to determine, through a comparison of direct total counts and viable counts, whether the population as a whole is affected in the same manner. At low temperatures, however, *e.g.* 22° C., it is evident that disinfection does take place at pH 4.9 when the transfer is made from a medium of pH 6.9 at 37° C. (Fig. 1, A). At higher temperatures, also, *e.g.* 46.1° C., disinfection is accelerated at pH 4.9 in comparison with the rate at pH 6.9 (Table I). It would appear likely, therefore, that disinfection occurs at pH 4.9 and 37° C. as well, although obscured by a simultaneous growth of a portion of the population. The fact that the growth rate returns to normal, however, suggests that some adjustment to the acid medium takes place within the organisms under these conditions. At the slightly higher temperature of 43° C., growth apparently continues at a logarithmic rate, more slowly at pH 4.9 than 6.9, and at both pH values considerably more slowly than at 37° C.

In accordance with results of the previous studies (1), Fig. 1, A, B and C show that the action of a given concentration of quinine at a pH near neutrality is strongly dependent upon the temperature.

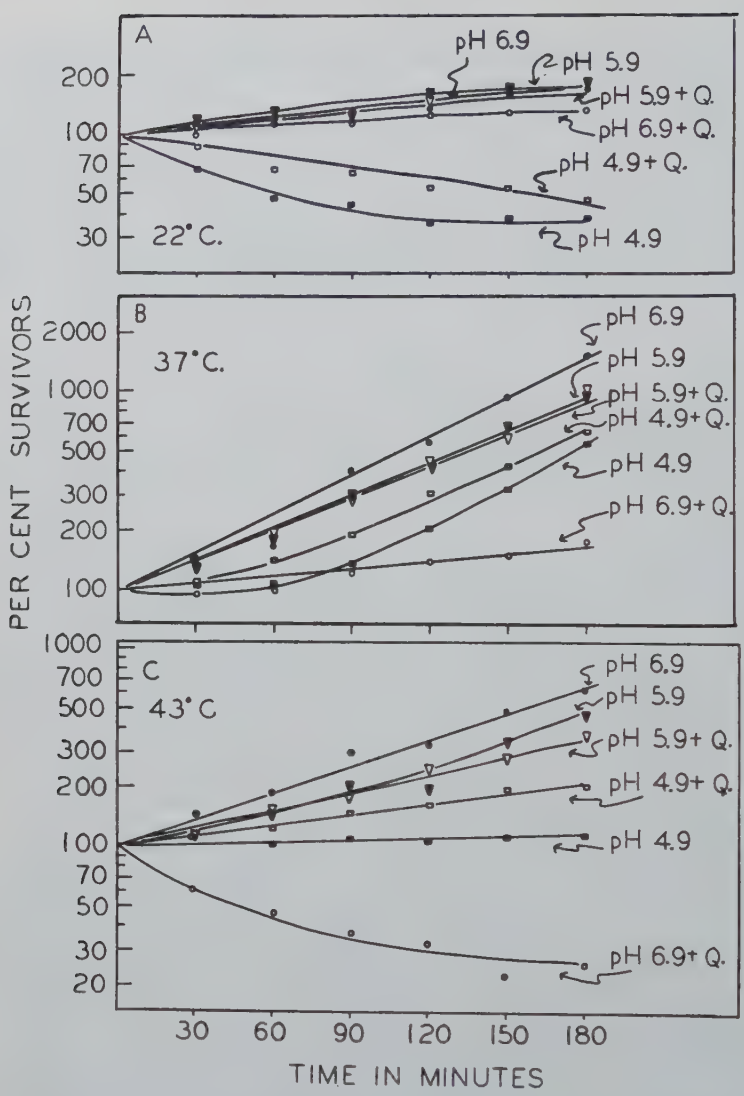

Fig. 1 A, B and C. Change in total number of viable cells with time, in media of pH 4.9, 5.9 and 6.9, respectively, after inoculation from a culture in the early logarithmic growth phase at 37° C. and pH 6.9. Per cent survivors is plotted on the logarithmic scale of the ordinate against time on the abscissa. Solid points: without quinine; hollow points: with 0.0007 molar quinine (Q). A, 22° C.; B, 37° C.; C, 43° C.

Table I.

Rate of disinfection at 46.1° C. in relation to pH and quinine.

The constant, k, represents the difference between the logarithm of the number of cells at the start and the logarithm of the number at the end of three hours, divided by the time, 10,800 seconds.

pH at start	pH after 3 hrs	molar concentration quinine	$k \times 10^7$
4.91	4.90	0	993
4.93	4.93	.0007	146
5.73	5.73	0	130
5.74	5.73	.0007	154
6.72	6.68	0	153
6.76	6.69	.0007	2610

These figures show that it is also strongly dependent upon pH at a given temperature, and serve to emphasize the necessity of using well buffered solutions in studies of the drug's action. The inhibitory effects of quinine on the rate of growth are practically eliminated by changing the pH only 1 unit, *i.e.*, from 6.9 to 5.9. Similarly, in disinfection, although quinine brings about a fairly rapid rate of death when the reaction of the medium is nearly neutral, it has very little effect if the pH is lowered to 5.73 (Table I).

These results indicate that it is the undissociated, or free base of the alkaloid that is responsible for the growth-inhibitory and disinfection-promoting action of quinine. A similar relation has been found with respect to the inhibitory effects of quinine on specific enzyme reactions, *e.g.*, those of invertase (11), serum lipase (12), and stomach lipase (13) as well as on more complex processes, including respiration and fermentation in living yeast cells (14, 15), and viability of protozoa (16, 17). Quantitative correlations between the concentration of the free base and the amount of inhibition or other effects of the drug are not fully justified in the present instance, both because of the fact that some of the rates undergo considerable change during the course of the experiment, and because accurate information concerning the dissociation constants of quinine under the conditions employed is not available [1]). Qualitatively, there is no doubt that the effect-

[1]) The constant for the first dissociation, at low temperatures, between 15 and 18° C., is given by Veley (18) as 2.16×10^{-7}, by Barrat (19) as 2.6×10^{-6}, and by Kolthoff (20) as 1.08×10^{-6}.

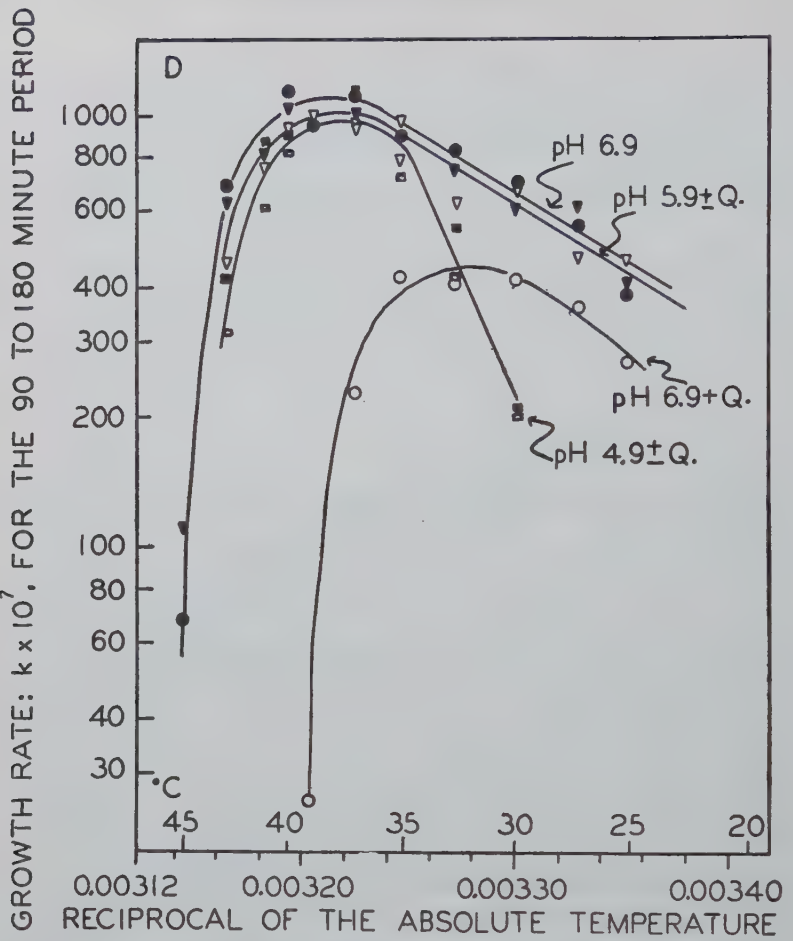

Fig. 1 D. The relation between temperature and rate of growth in media of pH 4.9, 5.9, and 6.9, respectively, with and without 0.0007 M quinine, respectively, during the period between 90 and 180 minutes after inoculation. The apparent rate constant, k, plotted on the logarithmic scale of the ordinate, represents the logarithm of number of cells at 180 minutes, minus the logarithm of the number of cells at 90 minutes after inoculation, divided by 5,400 seconds.

iveness of the drug increases with rise in pH, in the same direction as the increase in free base

In contrast to the inhibitory effects of quinine itself, under certain conditions the drug apparently opposes the growth-inhibitory effects of acid. This phenomenon was encountered during the initial period of growth, during the first 90 minutes at pH 4.9, throughout the temperature range where an increase in total number of viable

cells took place. Moreover, at this pH, the addition of quinine regularly retarded, rather than accelerated, the rate of disinfection at both low and high temperatures. With respect to growth at temperatures below the optimum such action is temporary. In disinfection at 46.1° C., the apparent „protective" influence of the drug persists more or less uniformly throughout the period of three hours.

In the respiration of yeast it has been shown that, on the acid side of the optimum, the same concentration of quinine which produces an inhibition at a more alkaline pH, leads to an increase in respiration at the acid pH, a phenomenon resembling the one discussed above in relation to growth (14). Accelerating effects of small concentrations of the drug have been encountered with other processes and enzyme reactions (21–24). The mechanism is not clear, and is not necessarily similar in each case.

The results of a number of experiments similar to, and including those illustrated in Fig. 1, A, B, and C are summarized in an analytical plot of the data concerning the rate of growth during the second 90 minutes of the experiment, as shown in Fig. 1, D. The lag in growth at pH 4.9 generally disappears by the beginning of this period of time, and the ensuing rate of reproduction is sufficiently nearly logarithmic to permit the calculation of an apparent rate constant. Some of the points in Fig. 1, D, are rather scattered, but it seems that the decrease in growth rate at this pH is now somewhat greater in the presence of the drug. The decrease in inhibitory effectiveness of quinine at a pH of 5.9 and below, in comparison with pH 6.9 is again clear, and also the increase in potency, at the latter pH, with rise in temperature.

With regard to the influence of pH on the rate of growth in the absence of quinine, it is of interest to note that the apparent activation energy is greatly increased by lowering the pH from 6.9 or 5.9 to 4.9. According to the slopes of the lines in Fig. 1D, the change in activation energy is from approximately 15,000 to 63,000 calories. The latter seems extremely high, and is probably influenced in this direction by the initial lag which, at low temperatures, is so prominent at the acid pH. In luminescence, also, there is a large increase in apparent activation energy at a pH of 5 in comparison to that at pH 7, (8), amounting to about 20,000 calories, the difference between approximately 40,000 at the former pH and 20,000 at the latter. In luminescence there is reason to believe that this difference represents the heat of dissociation of a hydrogen

on preceding the transfer of an electron in the oxidation of the reduced form of a flavoprotein-like enzyme responsible for light emission. Since the determination of apparent activation energies in the present instance is complicated by such factors as changing rates of growth, and the initially disinfecting action of pH 4.9 at low temperatures, the above figures relative to growth probably involve large errors. The difference in apparent activation energies at the two pH values, however, is in the same direction as in luminescence. Furthermore, inasmuch as the activity of a single enzyme under corresponding conditions but at neutral pH is apparently chiefly responsible for the rate of reproduction between 18 and 45° C. (1), a similar mechanism is possibly responsible for these phenomena in both growth and luminescence.

A more extensive study of the relations between pH, temperature and rate of growth, under both normal and increased hydrostatic pressure should provide further data significant to understanding the fundamental mechanism of the reactions that limit the rate of this process. The same applies to the process of disinfection at the relatively high temperatures, where the rate is more uniform, and not complicated by changes such as those of the lag in growth. Such changing rates require clarification before a satisfactory analysis may be achieved.

Summary.

The influence of pH in relation to temperature and 0.0007 molar quinine on the rates of growth and disinfection of *E. coli* in the logarithmic growth phase in a „synthetic" medium has been investigated.

The growth-inhibitory and disinfection-promoting action of quinine is greatly lessened by changing the pH of the medium from approximately 6.9 to 5.9, in the same direction as the decrease in free base of the drug at the lower pH.

At pH 4.9, an initial disinfection or a lag in growth takes place at low temperatures when the inoculation is made from a culture at 37° C. and pH 6.9. This phenomenon is less marked in the presence of quinine.

At relatively high temperatures, *e.g.*, 46.1° C., the rate of disinfection is much greater at pH 4.9 than at pH 6.9, and the addition of 0.0007 molar quinine considerably retards the rate of disinfection at pH 4.9. Although the disinfection-promoting action of quinine

in neutral solution is much greater at this than at lower temperatures, it is only very slight at pH 5.9 or 4.9.

The rate of growth at pH 4.9, following the initial lag, is apparently limited by reactions having a much higher activation energy than at pH 6.9.

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(Department of Bacteriology and Experimental Pathology, School of Medicine, Stanford University, California).

OXIDATIVE ASSIMILATION BY VARIOUS MICROORGANISMS

by

C. E. CLIFTON

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I. Introduction.

It has been generally assumed that the oxidation of foodstuffs makes energy available to the cell for synthetic processes, yet the energy coupling between the assimilatory and dissimilatory processes is difficult to picture in either physical or chemical terms. In 1930 Kluyver (1) in a discussion of the general problem of assimilation stated „— apart from hydrolysis and its reversion — the whole of biochemistry, the complex of all chemical changes brought about by living cells, can be reduced to chains of voluntary primary reactions, each of which consists in a coupled dehydrogenation." Examples were given which indicated that anabolic reactions may occur spontaneously, if the proper initial substances or building blocks are provided by the catabolic activities of the cell. These suggestions led to the concept that the dissimilatory processes may well serve as a source of raw material for the assimilatory processes rather than as a source of energy.

Kluyver's pioneer concepts in this field have been well supported by a number of studies in recent years (reviewed by Clifton (2)). The first important contribution concerning a stoichiometric rather than an energetic relationship between assimilation and dissimilation was made by Barker in 1936. Barker (3) reported that the colorless alga, *Prototheca zopfii*, apparently assimilated 50 to 80 per cent of the carbon present in simple foodstuffs such as acetate, ethyl alcohol and glycerol with the formation of primary building material of the empirical composition of a carbohydrate. The amount of carbon assimilated, as determined from balance sheets derived from amounts of substrate utilized, of oxygen consumed, and of carbon dioxide produced, was constant

for a given substrate but varied from one substrate to another. Similar studies by GIESBERGER (4) and CLIFTON (5) in KLUYVER's laboratory suggested that this phenomenon was of quite general occurrence amongst microorganisms but that the percentage of assimilation from a given foodstuff varied with the nature of the organism. The latter author also demonstrated that complete oxidation of the foodstuff without apparent formation of assimilatory products may occur in the presence of suitable cellular poisons, particularly sodium azide or 2,4-dinitrophenol. The studies of other investigators (see 2) confirmed the formation of a primary assimilation product of the empirical composition CH_2O from a wide variety of substrates by other microorganisms. In all instances a definite quantitative ratio was observed between the amount of oxygen required for complete combustion and that actually consumed or between the theoretical amount of carbon dioxide produced on complete combustion and that actually liberated by a given species utilizing a given substrate.

The concept that the dissimilatory processes may serve primarily as a source of building material was further advanced by CLIFTON and LOGAN (6) who demonstrated that the extent of assimilation is dependent on the chemical constitution of the foodstuff rather than on its available free energy content. They found that the same amount of carbon was assimilated from lactate as from pyruvate or from succinate as from fumarate, even though the latter compound in each pair has a higher free energy content. Also only one carbon atom was assimilated by *Escherichia coli* per molecule from any of the compounds mentioned above, although the energy content of succinate is markedly greater than that of lactate. DOUDOROFF's (7) observations with *Pseudomonas saccharophilia* and those of ANDERSON (8) with *Prototheca zopfii* also indicate that assimilation is dependent on chemical mechanisms concerned with intermediate products of dissimilation rather than on an energetic coupling of anabolic and catabolic reactions.

In the earlier studies on oxidative assimilation the extent of assimilation was based primarily on gaseous or heat exchange studies. WINZLER (9) demonstrated an increase in the reducing sugar content of yeast during the course of the oxidative assimilation of acetate while VAN NIEL and ANDERSON (10) observed an increase in the dry weight of yeast during the course of fermentative assimilation of glucose. PICKETT and CLIFTON (11) observed that while manometric studies suggest that the oxidative assimilation

of glucose by *Saccharomyces cerevisiae* may be represented as

$$C_6H_{12}O_6 + 2O_2 \rightarrow 4\,(CH_2O) + 2CO_2 + 2H_2O;$$

yet the observed increase in hydrolyzable carbohydrate, dry weight or carbon content of the yeast cells was only about one-half that predicted by the above equation. The discrepancy between the observed increase in carbon content of the cells and that postulated by the above equation was accounted for as organic matter other than glucose in the medium. The complete validity of these results is subject to question in the light of certain studies to be reported here. Certain of PICKETT's and CLIFTON's (11) results were based on analyses of control cells at the beginning of an experiment but it has become evident with improvement in technic that yeast cells in particular may release a considerable quantity of organic matter into their environment on shaking in Warburg flasks. This paper summarizes results obtained in a broader quantitative study of the quantitative relationships between the assimilatory and dissimilatory processes.

2. METHODS.

Washed suspensions of *Prototheca zopfii*, of *Saccharomyces cerevisiae* and of *Escherichia coli* were employed in this study, these organisms being selected as representative of the various groups of microorganisms employed in studies on assimilation. Oxygen consumption and carbon dioxide production were determined by the Warburg technic. Carbon analyses by the method of VAN SLYKE and FOLCH (12) were carried out on samples of cells and of supernatant fluid obtained on centrifugation of the contents of the Warburg vessels. Samples from the Warburg vessels were centrifuged in centrifuge combustion tubes and a measured portion of the supernatant fluid transferred to an ordinary VAN SLYKE combustion tube. These samples were evaporated to dryness in an evacuated dessicator before carbon determinations were made. All samples were acidified with sulfuric acid and evacuated to remove carbon dioxide before carbon determinations were carried out. As much as possible of the remaining supernatant fluid in the centrifuge tubes was removed without disturbing the cells. The cells were then washed with 10 ml of slightly acidified water and centrifuged from the suspension. One ml of 2 % barium chloride and 2 ml of 5 % sulfuric acid was then added to each tube and the precipitate of barium sulfate deposited by centri-

fugation on the packed cells. This film of barium sulfate over the layer of cells greatly facilitated the removal of the supernatant fluid without disturbing the cells. When *P. zopfii* was employed it was necessary to add a small amount of the detergent „Aerosol" to the suspensions in the Warburg vessels and in the centrifuge combustion tubes to reduce the tendency of the organism to creep in a film of water over the surface of the vessels. Control experiments indicated that this agent had no effect on the metabolism of *P. zopfii*.

3. Oxidative assimilation by *Saccharomyces cerevisiae*.

In a typical experiment (M/15 phosphate buffer, pH 6.0, 30° C.) on the oxidative assimilation of glucose by *S. cerevisiae*, 429 mm^3 of oxygen was consumed by the time that the rate of oxidation had decreased to a level approximately one-fourth of the maximum rate observed and 459 mm^3 of carbon dioxide was produced from 0.1 ml of M 10 glucose. This corresponds to an oxygen consumption of 32 % and of carbon dioxide production of 34 % of the theoretical values for complete combustion and suggests that the oxidative assimilation of glucose may be represented as

$$C_6H_{12}O_6 + 2O_2 \rightarrow 4\,(CH_2O) + 2CO_2 + 2H_2O$$

Carbon determinations were carried out in duplicate on the original cells and on cells from the Warburg experiments, with and without added substrate, and also with samples of the supernatant fluids. The original cells had a carbon content of 2.48 mg while the cells of the control suspension at the termination of the experiment contained 2.26 mg, a decrease of 0.22 mg which was accounted for in part as an increase in extracellular carbon of 0.15 mg and of 0.02 mg endogenous respiratory carbon dioxide. The observed increases in carbon content of the suspension medium in separate experiments appeared to vary with the length of the shaking period, age of the cells and with other factors as yet undetermined. It is assumed, perhaps unwarrantedly, that the decrease in intracellular carbon occurs to approximately the same extent with actively respiring cells. The balance sheets for carbon recovery support the validity of this assumption.

The average carbon content of the cells after oxidative assimilation of 0.72 mg of glucose carbon was 2.64 in the cells from Warburg vessels containing alkali in the center wells and 2.63 in the vessels employed for carbon dioxide determination. This

represents an increase in carbon content above that of the endogenous control suspension of 0.38 mg C as compared with 0.48 mg C postulated by the above equation. The carbon content of the medium was 0.05 mg greater than in the blank suspension and this together with 0.25 mg carbon in carbon dioxide and 0.38 mg increase in cellular carbon accounts for 0.68 mg C from the 0.72 mg of glucose carbon added to the Warburg vessels.

Similar results were obtained during the oxidative assimilation of 0.72 mg of fructose, sucrose or maltose carbon. Results of a comparative experiment are summarized in Table I.

Table I.

The oxidative assimilation of carbohydrates by *S. cerevisiae*.

Carbohydrate (0.72 mg C)	Increase in Cellular C	CO_2-C	Total C Recovered
Glucose	0.33	0.34	0.67
Fructose	0.43	0.27	0.70
Sucrose	0.46	0.29	0.75
Maltose	0.50	0.22	0.72

The increase in cellular carbon of *S. cerevisiae* observed with glucose in this experiment is undoubtedly low and may be high for maltose, as judged by increases observed in separate experiments. In a number of experiments the amounts of carbon assimilated from fructose, sucrose, or maltose tended to be greater than that assimilated from glucose while the extent and rate of oxygen consumption tended to increase in the order maltose - sucrose - fructose - glucose. This behavior merits further study.

4. Oxidative assimilation by *Prototheca zopfii* and by *Escherichia coli*.

As indicated under methods, the quantitative transfer of suspensions of *P. zopfii* was rendered difficult by the tendency of this organism to spread through films of water. Hence, the results obtained with this organism are subject to greater error. In an experiment attended with least experimental difficulty the increase in cellular carbon was 0.31 mg as compared with 0.36 mg postulated from manometric studies on the oxidative assimilation of glycerol.

Results reported by Clifton (2) on the oxidative assimilation of glycerol by *E. coli* show an increase in cellular carbon ranging from 0.13 to 0.15 mg as compared with the postulated assimilation

of 0.16 mg from 0.43 mg glycerol carbon. With glucose as the respiration substrate, the increase in carbon content of the cells ranged from 0.14 to 0.18 mg as compared with 0.23 mg postulated from 0.70 mg glucose carbon. Essentially similar results have been obtained during the growth of *E. coli* in a glycerol or glucose medium (unpublished results, see also CLIFTON and LOGAN (6) and WHELTON and DOUDOROFF (13)).

5. Discussion.

The results of quantitative studies on oxygen consumption, carbon dioxide production and carbon content of cells and suspension medium during the course of the oxidative assimilation of relatively simple organic molecules by representative microorganisms have been briefly summarized. The observed increases in cellular carbon approximate those postulated by the equations based on manometric studies alone and lend further support to the concept of oxidative assimilation. It must be borne in mind that these equations represent the primary synthesis of cellular substance and do not take into account the secondary reactions which may occur.

It is becoming more apparent that strictly chemical mechanisms for assimilatory reactions may agree more closely with the experimental data than do mechanisms based primarily on energetic considerations. The oxidation of foodstuff may serve primarily as a source of primary building blocks for the numerous syntheses carried out by the cell and in time it may be possible to picture the anabolic reactions as a series of chemical reactions analogous to those involved in catabolism. Provided the proper initial intermediates, cells may, by chains of spontaneously occurring primary reactions aided by energy rich phosphate bonds, transphosphorylations, transaminations, transaminidations, etc., carry out all the syntheses necessary for growth without the necessity of energy transfer between unrelated reactions. From this view point the cell is not regarded as an energy transformer and hence the metabolic efficiency of the cell should not be considered on the basis of the free energy available from the oxidation of a foodstuff but rather on the amount of building material inherent in the conversion of that foodstuff. It is still impossible to construct a complete picture of the anabolic activities of the cell but KLUYVER's prophetic words in 1930 appear to be gaining more experimental support. All that has been achieved is but a limited insight into those pro-

cesses and we may conclude with KLUYVER (1) that „the true greatness of biochemistry manifests itself in the synthetical processes, which are the first condition for growth and multiplication".

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(New Jersey Agricultural Experiment Station, Rutgers University, New Brunswick, N.J., U.S.A.).

SULFATE REDUCTION AND THE ANAEROBIC CORROSION OF IRON [1])

by

ROBERT L. STARKEY

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The process of sulfate reduction by bacteria is unique in that only a small group of bacteria, indistinguishable from one another morphologically and having the same general physiological characteristics, can bring about the reaction. The wide distribution of these bacteria becomes apparent from the general occurrence of black iron sulfide in waterlogged soils, in sediments of bodies of water, and in marshes. The bacteria responsible for the reduction of sulfate to sulfide were first described in 1895 by BEIJERINCK. In his several communications he laid the substantial foundation of knowledge of the process of sulfate reduction. The superstructure was erected by subsequent investigators, foremost of which were Dutch scientists. These were investigators at the Technical University in Delft, or scientists who had studied at Delft. To Professors BEIJERINCK (2, 3, 4, 5) and KLUYVER (10) and to VAN DELDEN (16), ELION (7), BAARS (1), and VON WOLZOGEN KÜHR (17, 18, 19, 20), credit is due for establishing the morphological and physiological characteristics of the sulfate-reducing bacteria, their distribution, and importance in nature. Contributions by other scientists have served to make additions to the main structure and provide refinements and ornamentations but the edifice can still be recognized as the product of the architect and artisans of the Technical University.

Interest in this group of bacteria by the Dutch scientists is no indication that activity of the bacteria is confined to the Netherlands. On the contrary their activity is evident throughout the world, and occasionally has resulted in undesirable effects requiring

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control procedures to prevent the destructive effects of hydrogen sulfide, the principal product of the reduction process. The odor of hydrogen sulfide is obnoxious, and sulfide causes discoloration of lead paints, textiles, foods, and water, destroys animal and plant life, is corrosive to metals used as containers, conducting tubes, and supporting structures, and produces black precipitates of iron sulfide which foul industrial equipment. Recently, there has been widespread interest in the sulfate-reducing bacteria as a result of the observation of VON WOLZOGEN KÜHR (20) that the bacteria are responsible for a severe type of corrosion of iron which occurs under anaerobic conditions.

Physiologically the sulfate-reducing bacteria are of interest because they develop under strictly anaerobic conditions with sulfate as the specific hydrogen acceptor. They also utilize other incompletely oxidized inorganic sulfur compounds as well as elemental sulfur but their ability to effect the reduction of these materials is shared with various other microorganisms. In his lucid account of the mechanism of bacterial oxidations and reductions, KLUYVER (10) gave consideration to the process of sulfate reduction. It was concluded that the process most probably proceeds by steps, each stage of which involves the transfer of two hydrogen atoms. Four reactions were therefore presumed to be concerned since, for the complete reduction of sulfate, eight atoms are involved. The substances probably produced during reduction were given as follows:

$$H_2SO_4 \rightarrow H_2SO_3 \rightarrow H_2SO_2 \rightarrow H_2SO \rightarrow H_2S$$

Demonstration of the stepwise reduction of sulfate has never been established experimentally but this in no way detracts from the validity of the hypothesis. The explanation lies rather in the difficulty of demonstrating the presence of intermediate compounds in culture media supporting growth of the sulfate-reducing bacteria. The data in Table I indicate, for example, that the sums of sulfate- and sulfide-sulfur equal the total sulfur content of the medium irrespective of the amounts of sulfate reduced.

In the reduction of thiosulfate there is likewise lack of accumulation of intermediate products (Table II). With thiosulfate as the only hydrogen acceptor, all of the sulfur of the decomposed thiosulfate was recovered as sulfide. The results obtained where the medium contained both thiosulfate and sulfate provide a partial explanation for the lack of accumulation of intermediate products

Table I.

Products of reduction of sulfate by *Sporovibrio desulfuricans*.

Treatment	Incubation period, days	Sulfide–S mg	Sulfate–S mg	Sulfide–S +sulfate–S mg	Total S mg
Mesophilic culture					
Uninoculated .	–	0	38.9	38.9	39.1
Inoculated . .	3	1.8	36.6	38.4	38.6
Inoculated . .	4	28.4	9.5	37.9	37.5
Inoculated . .	4	37.6	0.3	37.9	36.1
Thermophilic culture					
Uninoculated .	–	0	39.7	39.7	40.3
Inoculated . .	1	1.4	39.5	40.9	41.2
Inoculated . .	2	6.9	33.2	40.1	39.8
Inoculated . .	2	26.5	13.9	40.4	38.5
Inoculated . .	5	37.5	0.8	38.3	37.9

Table II.

Production of sulfide from media containing thiosulfate or thiosulfate and sulfate.

Inoculation	Incubation period, days	Thiosulfate–S mg	Sulfate–S mg	Sulfide–S mg	Thiosulfate–S + sulfide–S mg	Thiosulfate–S + sulfate–S + sulfide–S mg
Medium containing thiosulfate						
Uninoculated	8	39.4	1.5	0	39.4	40.9
Inoculated . .	4	38.9	–	1.9	40.8	–
Inoculated . .	5	22.4	–	17.9	40.3	–
Inoculated . .	6	12.9	–	29.0	41.9	–
Inoculated . .	8	0.8	0.1	41.2	42.0	42.1
Medium containing thiosulfate and sulfate						
Uninoculated	8	39.8	39.5	0	39.8	79.3
Inoculated . .	4	37.9	–	2.6	40.5	–
Inoculated . .	5	19.6	–	21.1	40.7	–
Inoculated . .	6	6.4	–	38.4	44.8	–
Inoculated . .	8	2.1	28.4	47.7	49.8	78.2

during sulfate reduction. It was found that most of the thiosulfate was reduced before there was evidence of sulfate reduction. The fact that the sums of the thiosulfate-sulfur and sulfide-sulfur did not exceed the amounts of thiosulfate-sulfur initially added to the medium, until practically all of the thiosulfate had disappeared, indicates that thiosulfate was more readily reduced than sulfate. There is no evidence that thiosulfate is an intermediate product of sulfate reduction, but if the intermediates are as susceptible to attack as thiosulfate the reason why there is no accumulation of intermediate products during sulfate reduction, is that these products are more readily reduced than sulfate.

Notwithstanding the possibility that other inorganic sulfur compounds than sulfate can be reduced by the bacteria, the principal sulfur compound available to the organisms in nature is sulfate and from this comes most of the sulfide which accumulates in black muds of the ocean sediments and tidal marshes, the sulfides which accumulate in the Black Sea and other bodies of water, and a large portion of the sulfide in the deeper layers of heavy wet soils and present in the products which form on metallic iron during anaerobic corrosion. Wherever free oxygen is excluded, and there is decomposable organic matter and sulfate in moist or wet environments, one is likely to encounter sulfide produced through the reduction of sulfate. In the words of VON WOLZOGEN KÜHR (20), „sulfate reduction is one of the most commonly occurring and extensive microbiological processes on earth".

Recognition of the general occurrence of sulfide in the products which accumulate on iron undergoing corrosion in wet soils of Holland prompted VON WOLZOGEN KÜHR to propose a new theory of bacterial anaerobic corrosion of iron and steel. This theory has focused attention on the sulfate-reducing bacteria as agents of corrosion and has led to the accumulation of evidence that the corrosion process is widespread.

Briefly stated, the role of the bacteria in the anaerobic corrosion process is ascribed to their oxidation of the hydrogen which is released at the cathodic areas of the corroding metal and, through this reaction, depolarization of the electrochemical system. The removal of the cathodic hydrogen is made possible through sulfate reduction. The transformation is indicated by the following reactions:

$$8\ HOH \rightleftarrows 8\ H^{\cdot} + 8\ OH'$$
$$4\ Fe + 8\ H^{\cdot} \rightleftarrows 4\ Fe^{\cdot\cdot} + 8\ H$$
$$CaSO_4 + 8\ H \rightarrow H_2S + 2\ HOH + Ca(OH)_2 \text{ (The bacterial process)}$$
$$Fe^{\cdot\cdot} + H_2S \rightarrow FeS + 2\ H^{\cdot}$$
$$3\ Fe^{\cdot\cdot} + 6\ OH' \rightarrow 3\ Fe(OH)_2$$
$$\overline{4\ Fe + CaSO_4 + 4\ HOH \rightarrow FeS + 3\ Fe(OH)_2 + Ca(OH)_2}$$

Von Wolzogen Kühr marshalled an imposing amount of evidence to support his theory, and additional evidence has been obtained by numerous other investigators. By this process it was possible to explain the corrosion of iron in soils of neutral reaction, free from stray current electrolysis and free from galvanic couples, a condition under which the factor responsible for corrosion had been obscure.

The bacterial theory of anaerobic corrosion is based on the fact that conditions under which anaerobic corrosion occurs are favorable for development of the sulfate-reducing bacteria, that the products of corrosion are those that would be expected from development of the bacteria, and that the corrosion process is made possible through bacterial removal of hydrogen accumulating at the cathodic areas, a reaction necessary to effect depolarization.

Brief mention of some of the important evidence supporting the theory of von Wolzogen Kühr will serve to indicate the validity of his claims.

1. The corrosion process occurs where conditions are strongly reducing and free oxygen is excluded. Under laboratory conditions, corrosion of iron typical of that encountered in the field was obtained with complete anaerobiosis in solutions supporting development of sulfate-reducing bacteria (19, 20). The weight loss of steel specimens kept for 156 days in sterile culture medium was 0.85 mg/dm²/day whereas in similar medium inoculated with sulfate-reducing bacteria the weight loss was 16.6 mg/dm²/day (6). The redox potentials of aggressive soils were low (100 mv or less) and much below the potentials of non-aggressive soils (above 400 mv) (12). The fact that sulfide is present in the corrosion products and frequently also in the aggressive soils is further evidence of anaerobic conditions (20, 8, 6, 12).

2. Anaerobic corrosion occurs at reactions close to neutrality (20, 12). The reactions of aggressive soils coincide with the reactions of culture media favorable for development of the bacteria (pH 5.5 to 8.5).

3. Iron sulfide is an important constituent of the products of anaerobic corrosion and greater amounts are contained in the corrosion products than in the surrounding soil. The sulfide is the product of sulfate reduction (20, 6, 12).

4. The fact that no free hydrogen is evolved in the corrosion process (19, 20) indicates that corrosion is not due to direct attack of the iron by H_2S as would be the case according to the following reaction:

$$H_2S + Fe = FeS + H_2$$

In addition, many aggressive soils contain no free hydrogen sulfide. Furthermore, in case that corrosion was due to a reaction between H_2S and Fe the ratio of total iron to iron combined as FeS in the corrosion products would be 1, whereas, according to the reaction proposed for bacterial anaerobic corrosion the ratio would be 4. Analyses of corrosion products showed ratios between 2.4 and 3.4.

5. Sulfate-reducing bacteria are able to oxidize hydrogen. It was noted by VON WOLZOGEN KÜHR (20) that, during development of sulfate-reducing bacteria in an anaerobic system containing hydrogen gas, hydrogen disappeared. STEPHENSON and STICKLAND (13) found that resting cells of the bacteria were able to effect the oxidation of H_2 by sulfate according to the following reaction:

$$H_2SO_4 + 4\ H_2 = H_2S + 4\ H_2O$$

Additional culture tests indicated that sulfate-reducing bacteria are able not only to activate molecular hydrogen as shown by STEPHENSON and STICKLAND but also to grow by the energy released through oxidation of hydrogen by sulfate (12). Crude cultures and partially purified cultures of sulfate-reducing bacteria developed in a completely inorganic medium containing sulfate as the hydrogen acceptor, bicarbonate as the source of carbon, and hydrogen as the source of energy, and in this medium reduced sulfate to sulfide with oxidation of hydrogen. As shown by a summary of the results in Table III, there was reduction of sulfate and disappearance of hydrogen. The calculated ratios of sulfate-sulfur reduced to hydrogen oxidized varied between 3.0 and 6.7. According to the reaction for hydrogen oxidation by sulfate, the ratio should be 4.0:

$$4\ H_2 + CaSO_4 = H_2S + Ca(OH)_2 + 2\ H_2O$$

The agreement between the postulated and determined ratios are surprisingly close in view of the fact that the determinations were

Table III.

Hydrogen oxidation by sulfate reduction.

Experiment No.	Sulfate-S reduced mg/L	Hydrogen oxidized mg/L	Ratio Sulfate-S oxidized/H_2 oxidized
1	305	65.2	4.7
2	237	78.7	3.0
3	221	52.2	4.2
4	253	72.0	3.5
5	350	98.5	3.6
6	205	53.1	3.9
7	192	49.5	3.9
8	259	38.7	6.7
9	220	53.6	4.1
10	359	107.5	3.3
Ave.			4.1
Ave. exclusive of experiment 8			3.8

made under conditions which did not permit accurate estimation of the exact amounts of hydrogen oxidized and sulfate reduced and that mixed cultures were used in most of the tests. Further evidence regarding the utilization of hydrogen by the sulfate-reducing bacteria was obtained from tests in which steel wool was substituted in the inorganic culture medium for the hydrogen gas (12).

The removal of nascent or absorbed hydrogen from the metal surface is of principal concern, however, with regard to the ability of the bacteria to depolarize the system and cause corrosion. In this connection, HADLEY found by electrical measurements that steel becomes more strongly anodic under the influence of sulfate-reducing bacteria (9). This was interpreted as evidence of depolarization by removal of the cathodic hydrogen, in whatever state it existed on the metal surface.

6. Development of the sulfate-reducing bacteria generally depends on the presence of utilizable organic compounds of which there are many (1). Under laboratory conditions with specimens of iron or steel in canal water or medium supporting development of sulfate-reducing bacteria the metal corrodes for a time after which there is no further attack, unless the substrate is renewed (6, 20).

There is no doubt that sulfate-reducing bacteria develop more

rapidly on organic media than in mineral media with hydrogen as the source of energy. Under soil conditions striking examples of acceleration of corrosion by organic materials decomposing on the surface of steel pipes have been noted (8). It may seem anomalous, therefore, that whereas the bacteria can utilize hydrogen under anaerobic conditions in the absence of organic nutrients, the process of anaerobic corrosion in soil depends on the presence of decomposable organic matter, but these two factors can be reconciled. Under natural conditions growth of the sulfate-reducing bacteria depends on the development of various soil bacteria which produce anaerobic conditions through decomposition of organic matter in compact wet soils. The sulfate-reducing bacteria also develop on the organic matter which serves not only as a source of energy but as a buffering and poising agent of the soil reaction and redox potential, as well as a source of nitrogen and other mineral nutrients. Some of the cells of the sulfate-reducing bacteria may activate the cathodic hydrogen as resting cells. Other cells may grow at the expense of the hydrogen liberated from the corroding metal. The decomposable organic matter may therefore be necessary to provide favorable environmental conditions for the sulfate-reducing bacteria and a sufficient number of cells to initiate the anaerobic corrosion process. This being the case, it would not be an essential nutrient for the bacteria which transform the hydrogen.

The evidence included above is convincing proof of definite relationship of the sulfate-reducing bacteria to corrosion according to the concept of von Wolzogen Kühr. Furthermore, as indicated by Hadley-the bacteria may intensify corrosion by affecting the metal surface in ways other than by removal of hydrogen. There is not invariably severe corrosion under anaerobic conditions, however, nor does it seem likely that corrosion under anaerobic conditions is due exclusively to bacterial activation of the hydrogen formed at the cathodic regions of the metal. It was pointed out by von Wolzogen Kühr that hydrogen sulfide, which is one of the principal products of sulfate reduction, may itself be corrosive. Elemental sulfur may be found in soils containing sulfides, for it is produced through interactions between sulfide and ferric hydrate or oxygen. Corrosion of iron by sulfur is readily demonstrated.

Of greater significance is the fact that iron sulfide is corrosive, because it is a common constituent of anaerobic soils and is abundant in the products of anaerobic corrosion. Stumper (14) observed that there was acceleration of corrosion when iron sulfide

was kept in contact with iron in an electrolyte. When the iron specimen was connected to the iron sulfide by an iron wire and exposed in one per cent NaCl, corrosion was approximately four times as great as that of the control specimen during test periods of 4 and 8 days.

Oxygen concentration cells or differential aeration circuits may also be established by sulfate-reducing bacteria, and corrosion resulting from these effects may be difficult to distinguish from that caused by the process whereby hydrogen is utilized by sulfate reduction. If two steel electrodes are placed in two vessels in a solution of an electrolyte, and if one is aerated and the other kept free from oxygen, and if electrical connections are made between the electrodes and the electrolytes, the electrode in the unaerated solution will be anodic to the electrode in the aerated solution, and it will corrode more rapidly (15). Similar oxygen concentration cells are produced on the surface of iron buried in soil. Small or large areas of the metal differ in degree of aeration depending on the extent to which oxygen is excluded from the iron surface. Active development of microorganisms in material adhering tightly to the metal surface may produce strongly reducing conditions and set up a current between the reduced areas and adjacent aerated areas. The regions where the sulfate-reducing bacteria develop will be anodic and the reducing conditions will be intensified by production of hydrogen sulfide.

There may be long line effects as well as oxygen concentration cells in small spots. A section of pipe in an area of waterlogged anaerobic soil would be anodic to another section of the same pipe in an adjacent area of well aerated moist soil. The active development of sulfate-reducing bacteria in the anaerobic soil would favor the development of these long line oxygen concentration cells or differential aeration circuits. Some of the cases of corrosion encountered by the author and described as anaerobic corrosion may have been due in part at least to differential aeration circuits. The effect would be the same as by the process described by VON WOLZOGEN KÜHR. Hydrogen would be produced in the process but it would be evolved at the cathodes in the aerobic soil and changed to water by reaction with oxygen. Iron would be liberated from the metal surface at the anodes and transformed to iron sulfide and ferrous hydrate.

POMEROY (11) was of the opinion that equipment used in sewage treatment and disposal undergoes corrosion through development

of differential aeration circuits. Corrosion of iron was not always encountered in equipment used to treat sewage containing an abundance of H_2S, although frequently there was severe corrosion. It was contended that if oxygen could be kept from the system and the equipment could be insulated from metal which was not aerated, the sulfide would not cause appreciable corrosion.

Conditions have also been encountered in soils, similar to those noted by POMEROY with sewage, namely that severe corrosion is not invariably encountered even though the iron surface is exposed to strongly reducing conditions where there is active development of sulfate-reducing bacteria. In some cases steel specimens buried in soils or marine sediment black with FeS and sometimes containing free H_2S, showed comparatively slight corrosion. In one such case, specimens which had been buried in black sediment containing large numbers of sulfate-reducing bacteria and an abundance of FeS showed less corrosion than similar specimens kept submerged in water above the sediments or periodically submerged in water and exposed to the air. One specimen which was almost completely buried in the sediment for 262 days lost 6 mg/dm²/day whereas a similar specimen which was continuously submerged in water above the specimen corroded at the rate of 14 mg/dm² day. Another specimen which was completely buried in sediment off shore lost 10 mg dm² day whereas another specimen periodically submerged in water and exposed to the air during tide changes lost 63 mg dm² day. Even though the steel was exposed in locations which were entirely favorable for development of sulfate-reducing bacteria, corrosion was comparatively slight.

The results briefly discussed in this report indicate that, although sulfate-reducing bacteria are concerned with corrosion of iron and steel under anaerobic conditions, corrosion does not always take place where it would be expected according to the bacterial process of VON WOLZOGEN KÜHR. Relationship of the bacteria to corrosion can scarcely be doubted but there is need for additional information concerning the role of the bacteria under various environmental conditions in order to provide a basis whereby cases of corrosion due to the process described by VON WOLZOGEN KÜHR can be distinguished from those in which the loss of metal is due to other causes. Likewise, much remains to be learned regarding the physiology and morphology of the interesting group of sulfate-reducing bacteria first described more than fifty years ago by BEIJERINCK.

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(Colonial Microbiological Research Institute, Port of Spain, Trinidad B. W. I.).

A METHOD DEVELOPED FOR THE STUDY OF YEAST PRODUCTION WITH *TORULOPSIS UTILIS*

by

A. C. THAYSEN and MURIEL MORRIS

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In the early days of food yeast production at Teddington, England, the method employed was based on the standard process of baker's yeast manufacture though the usual *Saccharomyces* species was replaced by *Torulopsis utilis*. In this method a heavy concentration of yeast, some 10 per cent dry weight of the total quantity of sugar to be fermented, is introduced into a volume of wort, containing approximately .5 per cent of available carbohydrates and a suitable concentration of certain nutrient inorganic salts. The volume of this initial wort is approximately half of the contemplated final volume of wort.

After inoculation the initial wort is maintained at 30° C. and vigorously aerated. Following one hours incubation additional quantities of a stronger wort, containing between 6 and 7 per cent of available carbohydrates, are added continuously and at predetermined increasing volumes until, after 9 hours incubation, the total desired volume has been built up. During this building up process the wort is vigorously aerated and maintained at a suitable reaction.

Experience has shown that under such conditions a yield of dried yeast, equal to 60 per cent of the added available carbohydrates, can be secured.

If a detailed analysis be made of the rate of growth of the yeast cells under such conditions, certain interesting data emerge. These can conveniently be discussed with the help of the figures recorded in Table I. These data are taken from an actual fermentation in the Teddington pilot plant. The counting of the cells was done in a Gower haemacytometer and all the cells counted were taken as viable in view of the short interval between the beginning and the end of the experiment.

Table I.

Time	Hourly addition of a molasses of 6.4 % strength	Total volume of wort in gallons at the end of:	Density of cell population per ml of wort at the end of:	Total cell population at the end of:	Residual concentration of carbohydrate in wort at the end of:	Weight of yeast per 100 ml wort at the end of:
	gallons		$\times 10^6$	$\times 10^{12}$	%	g
0 hours	20	129	841	488	.17	.7958
end of 1st hour	4.5	129	1148	666	< .10	.9598
„ „ 2nd „	5.5	133.5	999	600	< .10	1.1826
„ „ 3rd „	6.5	139.0	1515	947	< .10	1.2136
„ „ 4th „	7.5	145.5	1300	851	.16	1.3306
„ „ 5th „	9.0	153.0	1732	1183	< .10	1.4376
„ „ 6th „	10.5	162.0	1875	1367	.14	1.5052
„ „ 7th „	12.5	172.5	1552	1205	.19	1.6398
„ „ 8th „	15.0	185	1945	1619	.13	1.8160
„ „ 9th „	—	200	1744	1570	.21	1.8984

Column 5 of this table shows that at the end of the experiment the total number of cells in the wort had increased slightly more than threefold over the number present initially. The increase in weight of yeast was 3.7 fold.

From hour to hour the increase in total numbers of cells in the wort was somewhat irregular. This may have been due in part to technical short comings and in part to actual variations in generation time of the cells at various stages of the experiment. By generation time is meant the number of hours, or minutes, which it takes for one cell to produce a fully grown mature daughter cell.

The generation time of microbial cultures can be calculated (BUCHANAN (1928)) by the formula given below, when the total population of a culture at the time chosen is known.

The formula

$$g = \frac{t \times \log 2}{\log b - \log B} \qquad (1)$$

assumes that: t represents the time, in hours, during which growth had taken place, B signifies the initial total number of cells present and b the final total population.

For a given species the generation time of a single cell might

be determined by direct microscopic observation, provided optimal conditions for its growth were created. For *Torulopsis utilis*, the organism used in food yeast production, this was done and it was found that in 8 separate determinations, involving the mother cell and members of three subsequent generations, the average generation time was 84 minutes.

A simple calculation will show that at this rate of reproduction a given initial number of yeast cells and possibly also a given weight of yeast, would multiply more than 64 fold in 9 hours, as against the 3 to 4 fold increase observed in the experiment analysed in Table I. It would appear, therefore, that under the conditions adopted in the experiment of Table I a very much smaller output of yeast per volume of plant was obtained than should be possible theoretically.

Before any useful attempt can be made to deduce conclusions

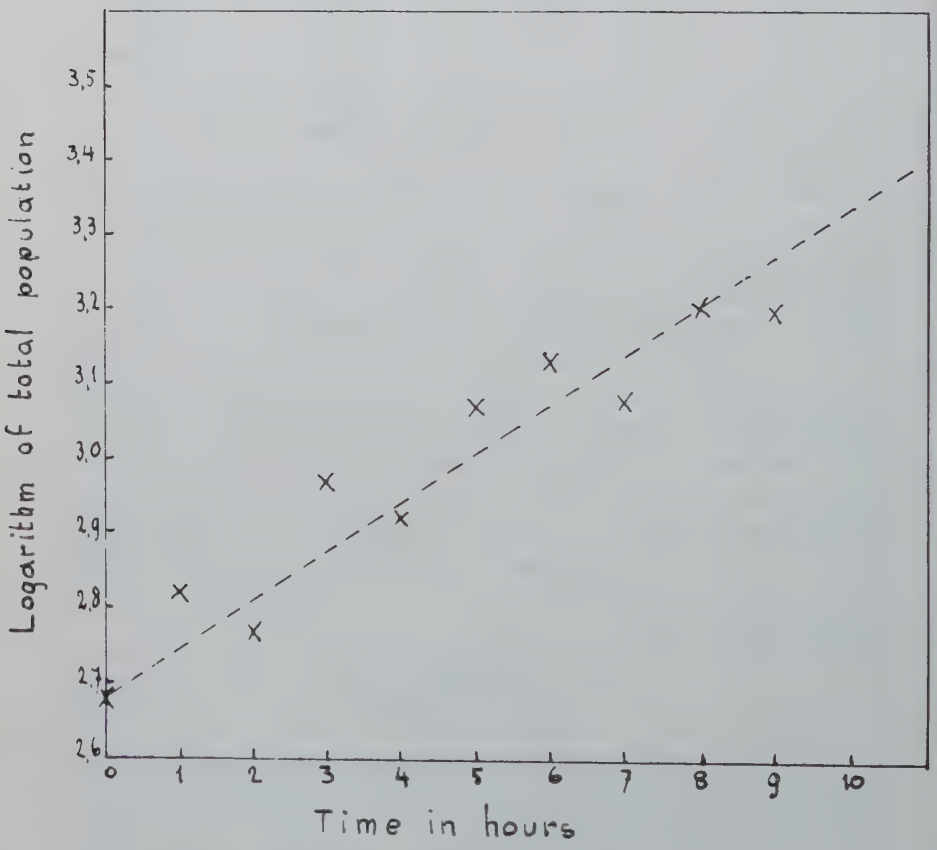

Fig. 1

of a technical interest from these observations it is necessary to study in some detail the generation times which were obtained in the experiment of Table I. This has been attempted in figure 1 in which the logs of the total population figures have been plotted against the time in hours of the experiment.

From these figures, and using BUCHANAN's formula the average generation time was calculated and found to amount to

$$g = \frac{9 \times 3010}{3.2700 - 2.6900} = 280 \text{ minutes.}$$

The wide discrepancy between this figure and that of 84 minutes recorded for *Torulopsis utilis* in growth tests under direct microscopic observation is not explained by any data contained in Table I, or for that matter by any observation gathered in carrying out the fermentation on which Table I is based. The only conclusion which it seems justifiable to draw is that conditions prevailing in experiment 1 must have been less favourable for growth than those existing in the culture of *Torulopsis utilis* observed microscopically.

Fortunately another experiment, carried out in the pilot plant at Teddington, throws some light on the causes of this discrepancy in rate of growth. This second experiment, which is analysed in Table II and Figure 2, is comparable in its general conditions with that of Table I both as regards aeration, reaction and food supply, but differs from the latter in the initial number of yeast cells present in the wort. This number was 48.8 times smaller than that of experiment 1.

Figure 2, in which the log of total populations is plotted against the hours during which growth proceeded, shows that the growth curve in this fermentation falls naturally into three fairly clearly defined sections, an initial period of slow growth, lasting about 2 hours, with a generation time of some 247 minutes, followed by an eight hour period of fast growth with a generation time of about 99 minutes. During the third period, covering 10 hours, the generation time rises again to 240 minutes. This third period approaches the average generation time of experiment 1 and probably represents the rate of yeast production under standard conditions of yeast manufacture since experiment 1 was carried out under such conditions.

In its main features the growth curve of experiment 2 embodies three of the stages of a microbial growth curve as defined by BUCHANAN (1928); the phase of positive growth acceleration,

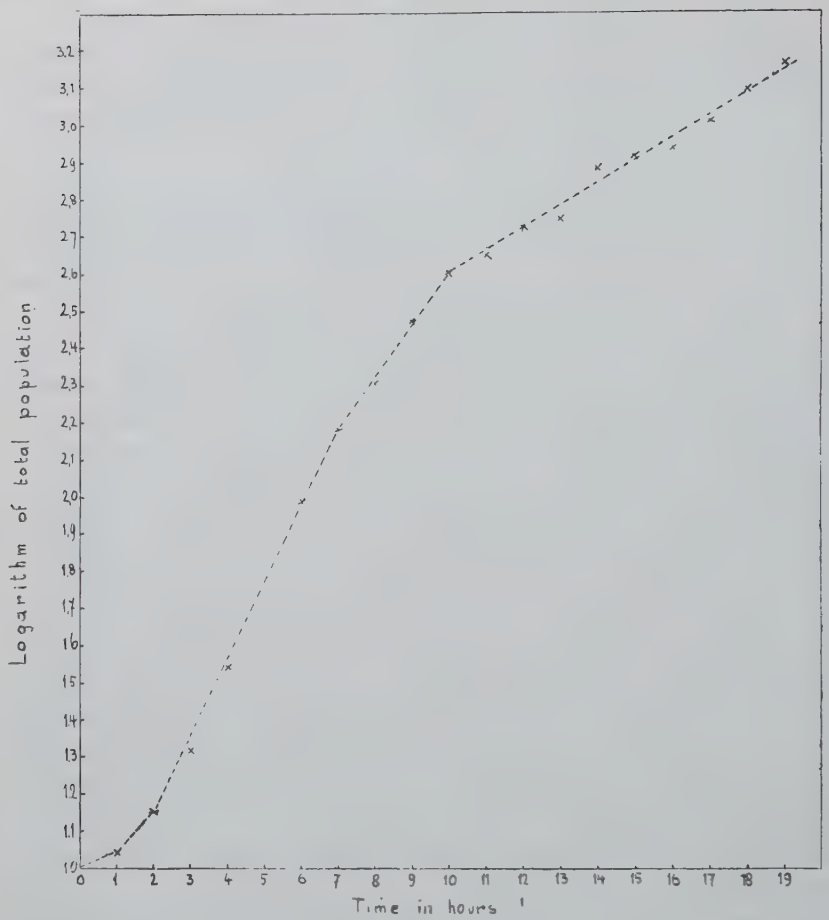

Fig. 2

between zero hour and the end of the second hour; the phase of logarithmic growth, between the beginning of the third and the end of the tenth; and part of the phase of negative growth acceleration, between the beginning of the eleventh and the end of the 19th hour.

It would appear, therefore, that growth conditions in commercial yeast manufacture are those prevailing during the period of negative growth acceleration, appart possibly from an initial brief period of positive growth acceleration.

The logarithmic phase of reproduction seems to have been completely suppressed with all that this must entail from a biological point of view, in loss of uniformity of age with consequent loss of

Table II.

Time	Hourly addition of a molasses solution of 6.4 % strength	Total volume of wort in gallons at the end of:	Density of cell population per ml of wort at the end of:	Total cell population at the end of:	Residual concentration of carbohydrates in wort at the end of:
	gallons		$\times 10^6$	$\times 10^{12}$	%
0 hours	27.3	100	17	10	1.4
end of 1st hour	—	125	20	11	1.39
,, ,, 2nd ,,	—	125	25	14	1.37
,, ,, 3rd ,,	—	125	37	21	1.35
,, ,, 4th ,,	—	125	62	35	.88
,, ,, 5th ,,	—	125	72	41	.67
,, ,, 6th ,,	—	125	173	97	.43
,, ,, 7th ,,	—	125	271	152	.16
,, ,, 8th ,,	—	125	357	201	.13
,, ,, 9th ,,	2.5	125	523	294	.13
,, ,, 10th ,,	3	127.5	691	397	.13
,, ,, 11th ,,	3.5	130.5	749	440	.15
,, ,, 12th ,,	4.5	134	873	526	.15
,, ,, 13th ,,	5.5	138.5	898	550	.16
,, ,, 14th ,,	6.5	144	1173	760	.17
,, ,, 15th ,,	8.5	150.5	1200	813	.20
,, ,, 16th ,,	9	159	1193	854	.23
,, ,, 17th ,,	12.5	168	1363	1030	.22
,, ,, 18th ,,	13.5	180.5	1520	1235	.22
,, ,, 19th ,,	—	194	1638	1430	.23

regularity of metabolic changes and uniformity of cell structure and cell content.

For this reason the standard procedure of yeast manufacture does not appear to be a particularly suitable one to adopt in food yeast manufacture, where a standardisation of the content of the individual cells, both as regards protein and vitamin content must be a primary consideration. Here a process based on the logarithmic period of growth would be preferable, provided its two most obvious shortcomings, its short duration and its apparent low density of cell population could be eliminated. An account will be given elsewhere of the work which made it possible to deal successfully with these difficulties. The brief note presented here merely outlines the principles on which the work was based.

Problems concerned with the extension of the logarithmic growth rate, or with the increase in the maximum density of yeast cells which can be maintained in a fermenting wort, can be studied experimentaly only with the help of theoretical fermentation schemes which it is possible to construct, when certain data are known.

How such a scheme is constructed will be outlined below and an example will be given which illustrates the degree to which an experimental fermentation can be conducted within the framework of the theoretical calculations.

In drawing up a theoretical fermentation scheme for the study of the extension of the logarithmic growth phase, allowance has to be made for the existence of an initial lag, or growth accelerating phase, during which each mother cell produces one fully grown daughter cell within four hours, or 240 minutes. Under such conditions the total number of cells present at the start in the fermenting liquid would double within four hours. As the lag period is assumed to end after three hours the total number of cells present at the end of three hours would be less than double the initial number. The actual numbers present after 1, 2 and 3 hours growth can be calculated from BUCHANAN's formula (1), already referred to, provided that the initial number of cells is known. The value for g in this case is known to be 4 hours.

After a lapse of three hours of slow growth the logarithmic rate of reproduction is assumed to set in and to continue for the remainder of the fermentation period. During this phase the generation time is taken as 100 minutes, so that a given number of cells will double every 100 minutes. It has already been mentioned that, under most favorable conditions, a generation time as short as 84 minutes had been observed. It was felt, however, that such highly favorable conditions might not be attainable under ordinary working conditions and that the somewhat slower rate of 100 minutes might be more easily attained.

With the aid of BUCHANAN's formula it is again possible to calculate the total number of cells which should be present theoretically at any given hour of the logarithmic growth phase since both the initial total number and the generation time are known. All these calculated date are set out in column 8 of Table III which has been constructed to illustrate a theoretical fermentation scheme in which the logarithmic phase of growth has been extended beyond its normal duration.

Table III.

Scheme for the study of the logarithmic rate of growth of *Torulopsis utilis* at a maximum cell population of 1000 million cells per ml.

Time	Total weight of yeast present at the end of the:	Hourly addition of available carbohydrates starting at the end of the:	Hourly addition of a 6% molasses solution starting at the end of the:	Hourly addition of sterile water starting at the end of the:	Total volume of wort at the end of the:	Density of cell population at the end of the:	Total cell population at the end of the:	Generation time
	g	g	ml	ml	ml	$\times 10^6$	$\times 10^{10}$	
0 hours	1.372	.454	8.3	—	1000	100	10	
end of 1st hour	1.644	.516	9.6	—	1000	120	12	240″
„ „ 2nd „	1.954	.612	11.2	—	1010	141	14.2	
„ „ 3rd „	2.322	2.126	38.6	—	1021	167	17	
„ „ 4th „	3.598	2.874	52.2	—	1060	242	25.6	
„ „ 5th „	5.322	4.71	85.6	—	1112	354	39.4	
„ „ 6th „	8.148	6.414	116.6	—	1198	499	59.8	
„ „ 7th „	11.998	10.964	199.2	—	1315	680	89.4	
„ „ 8th „	18.574	16.144	293.4	263	1514	893	135.2	100″
„ „ 9th „	28.26	24.164	439.2	624	2070	1000	207	
„ „ 10th „	42.76	36.6	665.2	1055	3134	1000	313.4	
„ „ 11th „	64.72	57.3	1041.4	1535	4854	1000	485.4	
„ „ 12th „	99.1	—	—	—	7430	1000	743	

For the purpose of calculating the amount of carbohydrates which would be required to maintain growth at the planned levels it is necessary to know both the weight of the yeast cells taken as inoculant, the hourly increase in weight of cells and the yield of dry yeast which can be obtained from a given quantity of carbohydrates. For purposes of calculation this third factor was assessed at 60 per cent, a figure which it was known could be reached when carrying out yeast production under the conditions outlined in Table I.

The weight of the cell population used as inoculant is not so easy to assess. Actual weighings differ considerably. In five seperate determinations the weight of 50×10^{10} cells of *Torulopsis utilis*, the yeast species used in food yeast production, varied between 5.875 g and 8.514 g with an average of 6.860 g, the figure finally adopted.

Table IV.

Experimental fermentation to show the logarithmic rate of growth of *Torulopsis utilis* with a maximum cell population of 1000 million cells per ml of fermenting wort.

Time	Total weight of yeast present at the end of:	Hourly addition of available carbohydrates starting at the end of:	Hourly addition of a 6 % molasses solution starting at the end of the:	Hourly addition of sterile water starting at the end of the:	Total volume of wort at the end of the:	Density of cell population at the end of the:	Total cell population at the end of the:	Generation time
	g	g	ml	ml	ml	$\times 10^6$	$\times 10^{10}$	
0 hours	.8700	.454	8.3	—	1000	99	9.9	
end of 1st hour	—	.516	9.6	—	1000	122	12.2	240″
,, ,, 2nd ,,	—	.612	11.2	—	1010	138	44	
,, ,, 3rd ,,	2.1197	2.126	38.6	—	1021	217	22	
,, ,, 4th ,,	—	2.874	52.2	—	1060	287	30	
,, ,, 5th ,,	—	4.71	85.6	—	1112	352	39	105″
,, ,, 6th ,,	7.2358	6.414	116.6	—	1198	463	56	
,, ,, 7th ,,	—	10.964	199.2	—	1315	680	89	
,, ,, 8th ,,	—	16.144	293.4	263	1514	1024	155	105″
,, ,, 9th ,,	27.8622	24.164	439.2	624	2070	1072	222	
,, ,, 10th ,,	—	36.60	665.2	1055	3134	932	292	
,, ,, 11th ,,	—	57.3	1041.4	1535	4854	1003	487	
,, ,, 12th ,,	91.8348	—	—	—	7430	975	724	

Though it is probably correct to say that the weight of a given number of cells of *Torulopsis utilis*, as of all other living cells, will vary at different periods of their life cycle, for the theoretical calculation it was assumed that the weight of yeast produced during growth would increase at the same rate as the cell populations, and that the weight of yeast, therefore, would double in four hours during the lag period and in 100 minutes during the logarithmic phase. The weight of yeast present at any given hour could, therefore, be calculated; it is shown in column 2 of Table III.

On the basis of the yeast weight figures, and on the assumption that 100 parts of carbohydrates would yield 60 parts of dry yeast it is possible to calculate the hourly carbohydrate requirements. These are set out in column 3 of the same table.

The corresponding figures in column 4 give the volumes of molasses wort, containing 6 per cent of available carbohydrates,

which would contain the calculated quantities of carbohydrates. In explanation of these latest figures it should be added that the molasses used in the actual fermentation contained 9.4 per cent of Fehling reducing substances which were unavailable for yeast growth.

Since it was intended to start the fermentation with an initial density of cells in the wort of 100 millions, the total volume of initial wort could be calculated by dividing the density of cells into the total initial population. The resulting figure is shown in the first line of column 6 Table III. The increase in volume of wort from hour to hour would be governed by the hourly addition of wort, at any rate until the end of the eighth hour. Since the density of population was planned not to exceed 1000 million cells per ml it was necessary, after the end of the eighth hour, to add increasing volumes of water, in addition to the molasses wort. The volumes of water required hour by hour can be arrived at by dividing the total cell population, expected at any given hour, by 1000 millions and by substracting from the resulting figure the volume of wort to be added during the hour in question.

An experimental fermentation carried out on the lines of the scheme outlined here is quoted in Table IV.

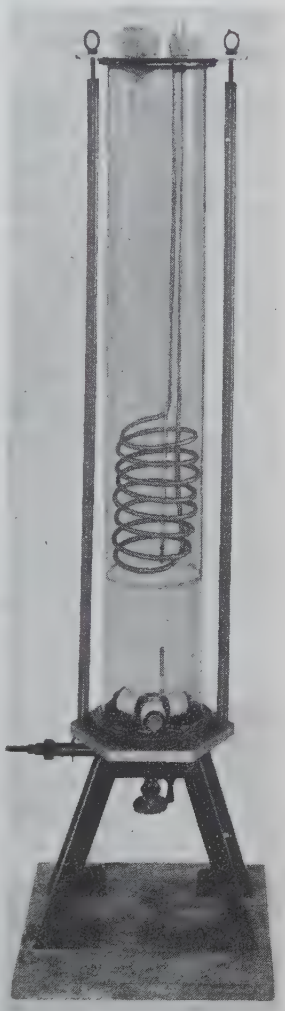

Fig. 3

The greatest care was taken to conduct the fermentation under optimal conditions, both in respect of temperature, which was maintained throughout at 30° C.; reaction, kept between pH 4.2 and 4.5; and aeration. The required air was supplied to the growing cells at a uniform rate through ceramic blocks, with apertures not exceeding 12 μ in diameter. Food substances, other than carbohydrates were added, either to the initial wort, or subsequently as required; nitrogen in the form of a 10 per cent solution

of ammonium sulphate and phosphorus as diammonium hydrogen phosphate. The phosphate was contained to the required amounts in the 6 per cent molasses solution used as wort.

The actual fermentation was carried out in a vessel designed for the purpose and illustrated in the photograph shown in figure 3.

Considering the experimental difficulties which are inherent in determining the density of a cell population by counting it in a haemacytometer, and those arising from maintaining optimal conditions in a fermentation under laboratory conditions, the results obtained (Table IV) are not unsatisfactory and show that a fermentation can be maintained in practice under conditions which conform closely to theoretical expectations.

Conclusions.

An account is given of the method adopted in the study of yeast production by *Torulopsis utilis*.

Literature.

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(From the Laboratory of Agricultural Microbiology, Faculty of Agronomy and Veterinary, University of Buenos Aires and the Laboratory of Microbiology, National Institute of Nutrition, Buenos Aires).

THE *FLEXIBACTERIALES* AND THEIR SYSTEMATIC POSITION

by

SANTOS SORIANO

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During the past few years, and in connection with previous investigations on *Azotobacter* species (7) and some higher filamentous bacteria of the order *Chlamydobacteriales* (4), the author had the opportunity to handle several strains of some particular forms of microorganisms characterized by a peculiar kind of creeping and gliding motility like that of the blue-green algae and the filamentous sulfur-bacteria of the genera *Oscillatoria* and *Beggiatoa*.

When the proper methods of isolation had been developed, a collection of 72 strains of this kind of strange organisms had been gathered from samples of mud and from pool and ditch water, and it was then possible to begin the study of their more important cultural, morphological and physiological characteristics in a pure state. Next to these and for purpose of comparison, 67 additional cultures have been secured (24 *Beggiatoa*, 36 *Cytophaga* and 7 *Myxococcus*), forming a total of 139.

The results of the observations accumulated since the discovery, made by chance, of the first strain of this natural group of microorganisms, are summarized in the present report, together with a brief account of the methods used during the different phases of the investigation and a short discussion upon what was considered the most appropriate approach to the establishment of their systematic position.

1. Methods of study.

Although a more detailed account of the methods of study employed during the first part of the investigation here reported,

has already been published (10), the most important steps will also be mentioned in this paper.

Enrichment: The *Flexibacter* and *Beggiatoa* strains were found usually sufficiently enriched when samples of surface waters with mud and decayed vegetable matter were kept occasionally for a few days in flasks at the laboratory.

The use of well developed enrichment cultures has been found necessary only for the *Cytophaga*; the best results being obtained by means of HUTCHINSON and CLAYTON's (5) nutrient solution.

Isolation: All cultures of the isolated *Flexibacter* species were obtained by a simple method, consisting in seeding the surface of 0,05 % meat extract (BUSGEN (3)) and 1 % agar, in Petri dishes with minute bits of material, wherein the presence of flexible bacteria had been previously ascertained, and the growth allowed to spread until it could be safely picked at the periphery, usually after incubation for some 12–24 hs at 28–30° C., preferably using any simple micromanipulator such as the one described by the author (6).

The *Beggiatoa* can be equally well isolated by this method, as has already been reported by CATALDI (4). As for the *Cytophaga*, the isolation is generally a difficult procedure, in which the use of glucose sterilized by filtration as stated by STANIER (11) has been usually found helpful.

Study of the pure cultures: The morphology and the motility of the cells have been observed more closely by simply placing a coverslip on the surface of the agar growth in Petri-dishes, and by dark field illumination of slide and cover-glass mountings. The staining has been performed with a hydroalcoholic-phenol-Bengale rose solution.

The size of the cells corresponding to the different species described in the text has been measured in unstained and living material grown on BUSGEN agar in Petri-dishes.

When in a pure state, the cultures grow fairly well in a 1 % peptone 1 % agar medium, but they have been maintained mainly in a soft (0.2 %) agar 0.1 % peptone medium, save the *Cytophaga* for which the addition of 0.2 % glucose (sterilized by filtration, or even by heat) has been found necessary, although they have been usually cultured in the liquid medium of HUTCHINSON and CLAYTON.

For the physiologic study a mineral medium like the one recommended by STANIER (12) has been employed using glucose and

filter paper as sources of carbon and several inorganic compounds as sources of nitrogen.

2. Description of some isolated forms.

The isolated cultures have been submitted to a careful microscopic control, especially by dark field illumination, in order to check their purity and their particular kind of motility. A part of the collection, formed by 67 cultures were strains of either *Beggiatoa*, *Cytophaga*, or *Myxococcus*, usually in a pure state.

Another part, formed by 72 strains, were identified as belonging to the new group of bacteria without sulfur granules, not digesting cellulose and not forming fruiting bodies on the substrate. Of these, 23 cultures have been examined more carefully and as a result of their study a genus and 5 species have been already differentiated, of which a brief description follows:

1. *Flexibacter flexilis* (type species): (6 strains). — Flexible, medium-sized and rather thick rods, with rounded, slightly pointed ends; 0,5—0,7 × 10—20 microns; edge of the growth on agar formed by masses of migrating cells, arranged in pointed columns continually moving in a strange clockwise direction, and forming loops. The cells must have a capsule for they do not come in complete contact with each other when moving. Motility not very active. Scanty growth in semisolid agar with extract of peptone. A beautiful „moiré", thin, and rapid spreading growth is formed on 1 % peptone agar.

2. *Flexibacter elegans* (4 strains). — Filamentous, thin rods, with rounded ends; 0,4—0,5 × 20—50 or more microns of length; very active flexibility and motility of the cells which often move with the twisted central part of the body going ahead, forming elegant, capricious figures, often spiraled; edge of the growth on surface of agar amorphous and a little curled; moderate growth in semisolid agar and good, yellowish growth on 1 % peptone agar.

3. *Flexibacter giganteus* (7 strains). — Rather thick organisms forming very long filaments of 0,75—0,9 × 100 and more microns of length. Rotatory, creeping and flexing, rather slow motility; often forming beautiful curled figures; edge of the surface growth on agar, curled, with strands of filaments usually running together, growth in semisolid agar is moderate, very finely granular, and forming clews looser and bigger in the deepest parts. Spreading growth on 1 % peptone agar, formed by entangled filaments, showing in certain strains a number of small elevations, especially

at the periphery. In test tubes of standard agar with yeast autolysate the growth takes usually an orange-reddish colour.

4. *Flexibacter albuminosus* (2 strains). — Rods, rather short and thin; 0,3—0,4 × 4—10 microns; flexibility of the cells not easily apparent; edges of the surface growth on agar, finely sawed, formed by numerous and short cellular lengthening; growth in semisolid agar rather abundant, white and albuminose-like. Good, thick, dirty-white growth on 1 % peptone agar, eventually forming a dark diffusible pigment.

5. *Flexibacter aureus* (4 strains). — Small rods, of 0,3—0,4 × 3—5 microns. Flexibility not easily apparent, due to the shortness of the cells; edges of the surface growth on agar, finely dented; growth in semi-solid agar rather abundant, white or yellowish. On 1 % peptone agar, the growth is good and abundant and takes a more or less deep orange colour.

The last two species are not easily differentiated. Some other 50 additional strains of this so called „yellow group" have also been studied along with the previous cultures, but the results could not yet been considered conclusive and will not be given in this report.

3. Systematic position.

The general appearance of the microorganisms here described, the absence in their cells of a synthetic pigment, and their behaviour on the various culture media studied during the investigation, strongly pointed to their classification among the bacteria.

The form of the cells, the absence of a sheath the lack of branching and the very remarkable character of their motility sufficed to exclude any of the following orders: *Actinomycetales*, *Chlamydobacteriales*, *Spirochaetales*, *Caulobacteriales* and *Rhodobacteriales*.

Some of the cultures, growing in very long filaments, with a gliding rotatory and creeping motility, and with the cells full of highly refractive (sulfur) granules had been identified as species of the genus *Beggiatoa* of the order *Thiobacteriales*, corresponding exactly to the strange and beautiful microorganisms isolated and studied by Cataldi (4).

Another strain, even before having been obtained in pure culture, was easily identified as belonging to the genus *Cytophaga*, due to its unmistakable origin from the enriched cultures being aerobic, cellulose digesting, flexible organisms, eventually proving to have the same potency in pure cultures.

The remaining cultures, which formed the greater part of the collection, could not be classified in any way among the other bacterial orders, so it was considered advisible to create for them a new genus named *Flexibacter*, and comparing them with the previously mentioned belonging to the genus *Cytophaga* and *Beggiatoa* it was considered advisible to group them under their respective families *Flexibacteriaceae*, *Cytophagaceae* and *Beggiatoaceae*, in a new order called *Flexibacteriales*, with the diagnosis given below (8, 9, 10).

Order *Flexibacteriales*: Bacteria of elongated or filamentous non-spiral cells; motility always creeping and gliding; do not form fruiting bodies on the substrate; resting forms, when present, are cysts; the cells may contain sulfur granules.

Three families are included in the order:

1. *Flexibacteriaceae*, with the genus *Flexibacter*, for the forms without sulfur granules and not digesting cellulose.
2. *Cytophagaceae* (STANIER 1940), for the cellulolytic forms, with the genus *Cytophaga* (WINOGRADSKY) and perhaps *Sporocytophaga* (STANIER).
3. *Beggiatoaceae*, with the genus *Beggiatoa* for the filamentous forms with sulfur granules.

STANIER (11) places the *Cytophagaceae* among the *Myxobacteriales* although they are not able to form fruiting bodies on the substrate.

As for the *Beggiatoaceae* they are described in the order *Thiobacteriales* in the 5th edition of the BERGEY's Manual (1), being apparently transferred to the *Chlamydobacteriales* (though they have no sheathed cells) in the new forthcoming 6th edition (BREED *et al.* (2)). STANIER and VAN NIEL (13) on phylogenetical considerations had for their part proposed to include them among their new division *Myxophita*, considering them not having a synthetic pigment as colourless counterparts of the blue-green algae *Cyanophyceae* of the genus *Oscillatoria*.

In the author's opinion the arrangement proposed in the present study establishing a new order for the three before mentioned families, has some practical advantages and it could be adopted without difficulty until a better understanding of the mutual systematic relationships among the different natural groups of bacteria may be experimentally attained at.

The relationship of the bacterial forms already described, with

other flexible bacteria, such as the myxobacteria and the spirochetes, and with the remaining bacteria with rigid cells, has led the author to present the following simplified dichotomous key for the classification of the bacterial orders, where the division in two main groups, namely *Eubacteria* and *Flexibacteria* as Sub-classes of the *Schizomycetes* was proposed (8, 9).

Class: *SCHIZOMYCETES.*

A. Cells rigid. Sub-Class I: *EUBACTERIA*
- I. Unsheathed:
  - 1. Non mycelial:
    - *a.* Non photosynthetic:
      - 0. Unstalked. . . Order I: *Eubacteriales*
      - 00. Stalked . . . Order II: *Caulobacteriales*
    - *b.* Photosynthetic . . Order III: *Rhodobacteriales*
  - 2. Mycelial. . . . . Order IV: *Actinomycetales*
- II. Sheathed . . . . . Order V: *Chlamydobacteriales*

B. Cells flexible. Sub-Class II: *FLEXIBACTERIA*
- I. Non spiral:
  - 1. Without fruiting bodies Order VI: *Flexibacteriales*
  - 2. With fruiting bodies . Order VII: *Myxobacteriales*
- II. Spiral . . . . . Order VIII: *Spirochaetales*

Summary.

From samples of ditch and pond waters, especially those containing decayed vegetable matter, some 100 cultures of a new group of bacteria have been isolated and studied in pure cultures. The most important characteristic common to all of them is the flexibility of their cells or filaments, showing a peculiar kind of creeping and flexible motility, similar to the one exhibited by the filamentous sulfur bacteria of the genus *Beggiatoa*, the aerobic cellulose digesting bacteria of the *Cytophaga* group, and the myxobacteria.

Five species corresponding to a genus, were briefly described, namely: *Flexibacter flexilis* (type species), *Fl. elegans*, *Fl. giganteus*, *Fl. albuminosus* and *Fl. aureus*; furthermore, the relationship with other bacterial cultures corresponding to the genera *Cytophaga* and *Beggiatoa* was established and the respective families *Flexibacteriaceae*, *Cytophagaceae* and *Beggiatoaceae* were then proposed to be placed in the new order *Flexibacteriales*.

1. *Flexibacter flexilis.* – 2. *Fl. elegans.* – 3. *Fl. albuminosus.* – 4. *Fl. aureus.* – 5. *Fl. giganteus.* – 6. *Beggiatoa alba.* – Magnification: × 1.000.

Coverslip preparations of the edges of 2 days growth on 1 % peptone agar (figs. 1 to 5) and on 0.05 % peptone (Busgen) agar (fig. 6), stained with carbol-Bengale-rose hydroalcoholic solution.

(Pictures by E. A. Padorno, Photographic Laboratory, Faculty of Agronomy and Vet., University of Buenos Aires).

Finally a simplified key for the classification of the bacterial orders generally accepted in the literature, together with the one proposed has been presented, the most important features of which are the establishment of two major taxonomic entities: *Eubacteria* and *Flexibacteria* as Sub-Classes of the *Schizomycetes* and the use of only one character at each step of its dichotomical divisions.

A plate of illustrations of the five described species of *Flexibacter* and of *Beggiatoa* alba is included.

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ON THE CULTIVATION OF FUNGI IN PURE CULTURE

by

JOH[A]. WESTERDIJK

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During the 19th century the morphology of the fungi has been intensively studied. In the fifties, however, mycologists also began to take up the biology of these organisms. So the French mycologists, the brothers TULASNE, attempted to make spores of Basidiomycetes germinate in water. Now and then they succeeded in keeping the water free from other organisms and thus were the first to enter the field of pure culture.

The botanist KLEBS as early as 1873 tried to grow bacteria in pure culture by means of continuous dilution of the mass of bacteria in boiled water; so did the physician LISTER who even obtained pure colonies by distributing drops of a very dilute suspension of bacteria over slices of potato. It was, however, the physician R. KOCH who introduced the actual pure culture by adding gelatine or agar to his solutions (1885).

As to the culturing of fungi on such media the botanist A. DE BARY went further. For the accomplishment of his work: „Vergleichende Morphologie und Biologie der Pilze" he has partly made use of pure cultures, but he mentions his methods very summarily. Had it not been for the oral communication of F. A. F. C. WENT, who in the eighties went to DE BARY to learn „how to cultivate fungi", I would certainly not have been aware of the advanced methods used by DE BARY. WENT learned from him the cultivation on nutrient gelatine and agar.

In the meantime E. C. HANSEN in Denmark was also working with pure culture of yeasts cultivated on beer wort. The study of fungi in pure culture has been greatly advanced by OSCAR BREFELD, botanist, whose works were published between 1872 and 1908, the later ones even after he had lost his sight. This solitary scientist, who apparently had little contact with his colleagues, has published

a wealth of observations bearing on many species of fungi. His method in the ordering of facts, which are treated much in detail and somewhat at length, is not very clear. Even in the volume „Neuere Kulturmethoden" (1880) it is difficult to distil from the stray notes what principles he has followed in his culturing. It is clear, however, that he has not been aware of a sterilisation over 100° C. and that he boiled his media some times in succession. It seems possible that he neither knew the methods of Koch with gelatine and agar media. He was, however, ahead of his time in as far as the nature of his nutrient media is concerned. The beer wort or malt, known already in the cultivation of yeasts, is among his usual media. He adds it as well to media of vegetable origin, *e.g.* to extracts of potatoes he adds drops of malt extract. Besides he was probably the first to use fruit juices, more especially juice of plums. On this plum juice he also successfully cultivated „mushrooms" and more especially those which develop on dead wood. A minute supply of extract of dung made these thrive better. He was already well aware of the fact that species of fungi occurring in humus develop but sparsely and do not fructificate on nutrient media. Striking as well are his experiences with species of *Nyctales*. Although he could induce the development of *Nyctales asterophora* on malt, he meets difficulty with *N. parasitica*, parasiting on caps of mushrooms. After adding a quickly dried cold extract of caps of *Russula*, an excellent germination of spores occurs. When, however, the caps of *Russula* have been boiled, this beneficial influence is lacking.

He cultivated parasites of insects on a broth prepared from the latter. The *Saprolegniae* parasitizing on water animals grow as well on such extracts. Also in this case he adds malt extract, again prepared without heating. *Phytophthora infectans* has been made to thrive on an extract of very young potato tubers, again after addition of malt.

So in his works many interesting examples may be found of cultures on „natural media", on extracts of plants, animals or soil particles. He generally cultivated in Erlenmeyer flasks and probably he has mainly used liquids; on the other hand bread was often imbibed with the extracts. He himself mentions his cultures as being not always pure by far. This faulty technique, however, led him to the conclusion that contaminating fungi did sometimes stimulate the growth of those in culture (synergism).

When in the 20th century the sterilization in autoclaves had

conquered the field everywhere, synthetic media then came much more into use, an innovation which proved far from ideal for most of the fungi in the long run. The notion „standard solution" has not proved appropriate for a continued cultivation, a fact which will be discussed further down. It is a sure fact that plant extracts and plant parts, such as seed, roots, etc. are to be preferred and nowadays these are more generally applied again.

I mean to offer here a short survey of the experiences gathered in the Centraal Bureau voor Schimmelcultures with various growth media. Although many of them are not based on extensive scientific experiments and it may be that a closer investigation would lead to other conclusions, it may be useful to make some communications after 40 years of „culturing of fungi". I have renounced from treating the group of yeasts, as these are being supervised by Professor KLUYVER, who is much more experienced in this group than I am.

Our collection comprises fungi of widely varied groups. The result is that also widely varied media have to be made use of. A medium ideal for all of the groups does not exist. The general experience is that species of fungi need to be cultivated alternatively on widely different media. Life asks for change unless the organisms are especially specialised. Rich media have to alternate with meagre ones, dry with moist, media from complicated substances with those from simple compounds. Mostly this principle is not sufficiently kept in mind. Once a good medium having been caught hold of, one is apt to keep to it, whilst in the long run the cultures will lose either their fructification or their healthy vegetative development. Degeneration is mentioned then, which may find its expression in a great variety of ways.

Another important factor which may lead to strong degeneration is the concentration of the carbohydrate which is generally taken too high. In many cases the synthetic media will contain 5—10% saccharose or glucose and such will be also the case when malt extract is used; even higher contents are not rare. We, however, use media with 2—3 % sugar. Thus the occurrence of globules of fat in the mycelia is prevented (*Mucor*) and the production of acid in the nutrient media is restricted. The higher the concentration of sugar the stronger is the autolysis due to too strong a formation of acid. Autolysis implies the need of transfer after much shorter periods. As for the pH, when kept between 3.5—7 it is of less influence on most fungi than might be expected. In a general

way the neutral reaction is to be preferred, notwithstanding fungi mostly are known as acidophilous. In fact, this is not correct. Series of succeeding pH's show clearly that even plant parasites may prefer extracts with a pH of 7.5 (*e.g. Nectria*). Actinomycetes very distinctly prefer an alkaline medium. Although nowadays these are actually reckoned among the bacteria, they make still part of the collection of the Centraalbureau.

The preference for vegetable extracts when compared with synthetic solutions has been pointed out already. In physiological investigations for the determination of metabolic products, the latter of course cannot be dispensed with. For continuous cultivation, however, they are quite inappropriate and apparently by far too simple in composition. So we hardly use the CZAPEK and RICHARDS solutions. The extracts of plantmaterial (bulbs, tubers, twigs, stalks and germinating seeds, malts) contain in fact an endless number of various substances, nutritive as well as auxines, as yet unfathomed. Our knowledge of the influence of auxines on the development of fungi, notwithstanding they are very much in the picture, is still limited. The influence of the extracts added by BREFELD in minute amounts will have partly to be ascribed to auxines and vitamines. The plant juices prepared from cherries, plums, tomatoes and other fruits are primarily suitable as media for isolation. They have a low pH (3—4) and thus prevent the development of many species of bacteria. But among these juices it is cherry juice which highly benefits the development of many species. BREFELD used the juice of plums; this juice, however, not merely favours yeast development in high measure and thus gets easily contaminated, but for instance *Basidiomycetes* are less healthy on this medium than on cherry juice. Most of the wood parasites (*Basidiomycetes* and other groups) thrive perfectly on this medium, as well as the organisms causing leaf spots and anthracnoses. The latter are parasites which develop merely small colonies on nutrient media; on the host plants they extend as well slightly. They act detrimentally on the host plant by their induction of numerous, densely crowded small infections (*Septoria, Cercospora, Phoma, Ascochyta*). This group of fungi thrives best on media of low concentration. No sugar may be added to the cherry juice.

The organisms causing rots on the other hand, ask for a medium richer in carbon food than the fruit juice can offer. Plant parts containing sugar such as roots of *Daucus carota* and *Beta*, bulbs containing starch, or grain of wheat, maize, barley (germinated

or ungerminated) fulfill these demands. Oatmeal is an excellent medium for those fungi which can put up with a rich nutrition. A peculiar source of starch which certainly will contain stimulating substances is saleb (finely ground tubers of indigenous orchids). Even added to beer wort, it may have a favourable influence.

The toxic substances the parasitism is based upon, the products of metabolism, in most cases are unknown. They arise in an acid medium and quickly decompose the tissue of the host. Sterilized plant parts are decomposed at a quick rate as well. As examples may be cited the main producers of sclerotia: the genera *Sclerotinia*, *Sclerotium*, *Botrytis*; also many Phycomycetes from the family of the *Peronosporaceae*. Soil saprophytes generally develop as well as the former parasites on vegetable substances. They usually prefer either starch, sugars or cellulose (*Dematiaceae*) and even lignine, such as *Mycena*, *Marasmius* and other fungi from the litter in forests. The latter thrive well on bran. The wide spread species of the genus *Fusarium*, parasites as well as saprophytes, are bound to grow on richer media (malt, oatmeal) but they sporulate often more normally on hard stalks. There is some drying up needed to give rise to sporodochia. But even then changing of media is necessary.

Still other groups exist which like *Fusarium* can convert cellulose and which also develop spores better on woody stalks than on agar. These are the *Dematiaceae*, imperfect forms such as *Alternaria*, *Stysanus*, *Stemphylium*. These species, however, may also be cultivated on very thin layers of agar, so that the mycelium very soon touches the glass wall. Oatmeal and cornmeal are very appropriate for this method.The nutrient agar is melted and the tube whilst being turned, is cooled under the watertap. A very thin layer is thus formed, upon which masses of spores will develop. A likewise abundant spore formation may be obtained by nutrition with cellulose; to this end a strip of filter paper imbibed with a weak nutrient solution, containing but little sugar, is useful.

Sometimes glycerol may be appropriate as a source of carbon, thus for parasitic Actinomycetes. It is added unto starch containing food (potato plugs).

As to nitrogen compounds, these occur in organic or inorganic form in sufficient amounts in plant extracts; in the synthetic media they are given as mineral compounds. For all organic nitrogen compounds we have to leave open whether such preparations act as a source of „nitrogen food" or that the favourable action is due

to definite auxines, which occur as „contaminators" such as for instance thiamine or its components. The same keeps true for carbon preparations in raw form (maltose brute).

Results obtained with RAULIN's solution show clearly that the combination of a great number of minerals with some metallic ions may stimulate the initial growth of fungi. Experience has taught, however, that for a lasting good development the vegetable substances with their components of widely varied character fulfill all demands, those for minerals as well.

For fungi, pathogenic to men (Dermatophytes) or other animal parasites organic compounds such as peptones are needed. These are also in use for parasitic Actinomycetes. It has to be kept in mind, however, that various brands of peptone act differently on organisms. Those that are least purified, are often the most favourable.

Strong parasites of plants such as *Venturia*, *Claviceps*, thrive better on asparagine as source of nitrogen, than on nitrates or compounds of ammonia.

Every one who cultivates fungi knows that many forms exist which as yet have never been made to sporulate in pure culture; it has been conceived, that this might be due to a changing of components of the medium caused by the high temperature during sterilization. Experiments, although still unpublished, have been carried out in which the media have been made free from germs by means of Seitz and other filters. Results, however, have been negative. Thus the filters either are too strongly adsorptive or they might produce substances inhibitory against the auxines which are necessary for a normal development. The slight knowledge about the action of redox potential on the intake of vitamines by fungi has not until now been applied in the cultivation of fungi for longer periods. Generally culture media are kept stored in the dark, although any certitude about action of light on auxines has not been obtained.

Next to the chemical composition of the medium we have to reckon with the influence of temperature during cultivation. The period in which it was believed that fungi just as bacteria would need a constant temperature, is long past. Experience has taught that the growth optima vary very much for different fungi and that for most of the fungi it is fairly low. Just as an alternation in media an alternation in temperature gives good results. The change from higher to lower temperatures may act favourably on

the forming of fruiting bodies by Basidiomycetes. The influence of an optimal constant temperature may induce the development of much aireal mycelium, a phenomenon which develops at the cost of the formation of spores. In the long run a fairly low room temperature is most suitable; it changes sufficiently in the various seasons. Even fungi, the optimum of which lies at 24—26° C. (such as many species of the genus *Fusarium*) develop well under such conditions. But rarely higher temperatures are needed: for the Dermatophytes, the thermophilous fungi (fungi of the self-heating of hay) and Aspergilli, especially *A. fumigatus* (50° C.) and for Actinomycetes which in many respects correspond with bacteria. Besides it has to be kept in mind that the formation of mycelium, of asexual and of sexual spores, each have an optimum of their own (*Sporodinia*). Perithecia often develop at higher temperature (*Penicillium*).

Narrowly connected with temperature is the humidity; a minimum of 7 % is required. A very moist stagnant atmosphere such as often occurs in cellars, induces moistness of cotton plugs and thus infections. Some groups thrive exceptionally well at a low moisture content (Aspergilli). Thus they develop well on media with a high osmotic concentration, for instance on jams.

As to the influence of light our experience is that generally it acts more stimulating than inhibiting. In many cases it is needed for the formation of pigment (Penicillia, *Neurospora*), in other cases it acts detrimentally. Often but not always light is the factor which gives rise to concentric growth rings with spore formation.

Pleospora (*Macrosporium*) merely forms conidia when illuminated more strongly than by diffuse light in a room. The same keeps true for *Helmimthosporium*. It is, however, remarkable that *Pleospora* develops perithecia without any extra supply of light. Neon light sometimes acts favourably on the production of spores; in short the action of light asks for much further study. Strong sunlight, the raise in temperature behind large window panes may in many cases cause too strong a drying of the cultures; especially for Phycomycetes this seems dangerous. Considering the markedly favourable action of light, a greenhouse which would be reasonably cooled during strong sunlight might prove more ideal surroundings for fungus cultures than a room. In a good culture of course normal development of all stages of the fungus is aimed at. This appears as yet not to be possible in many cases.

It is certain that each culture medium and all environmental conditions act in a definite way on each organism. On one species more than in another in the long run the size of the spores will change — even the shape may vary. Oval spores may grow nearly circular in agar media; other spores may grow smaller; walls may swell. At all events it is desirable that at the receipt of a culture its forms are reproduced in a drawing. Sectorial changes may occur which are hard to distinguish in tubes. Herefore as well it is necessary that the original form has been noted.

Moreover changes in the metabolism may or may not arise under the influence of external conditions. Thus the excretion products of fungi are very hard to garantee and would need a continuous chemical control. Examples of this are known since long; in the industrial preparation of antibiotica (penicillin), this phenomenon has been of very frequent occurrence.

When cultures for chemical conversions are demanded it will be needed to attempt at a selection of strains which will thrive well in a liquid. In one case thick surface layers may be needed, in another submerged development will be wished for. This depends on the occurrence of the desired metabolic products either in the mats or in the liquid. Of late attention has been drawn more and more to characteristics of various types of a single species which may be noticed in submerged mycelium. It looks as if the characteristics in submerged growth will need to be taken into account in the diagnosis of various groups of fungi.

Finally a few words on the subject of transferring. First of all it is desirable, whenever possible, to transfer from sporulating mycelium; fluffy aireal mycelium, when transferred will generally give rise merely to sterile growth. Such cultures are generally not healthy and show a strong degeneration. In some cases it will be needed (Basidiomycetes) to transfer fairly large pieces of the medium along with the fungus, as spores of many species have difficulty in starting their germination and the hyphae are very sensitive to the high temperature of the needle. For many species a transfer thrice a year is sufficient, but others ask for a renewal every two months, especially those which thrive best on media which dry up easily (roll cultures). The Oomycetes which very easily get contaminated with bacteria as well ask for about monthly transfer, *Chytridiaceae* for even shorter periods.

Various kinds of degeneration (deterioration) due to the medium have been pointed out already. Certainly more transfers than

may be realized in a collection and more changes in the external conditions would greatly benefit the fungi.

The storing of species in small tubes according to the lyophylic method of RAPER and ALEXANDER (freezing, followed by desiccation in vacuo) is convenient and spares much time and care. For hygrophilous species (certain Phycomycetes) this kind of storing has appeared impossible; much further study is needed, however. Though extremely useful in the case of transport of cultures, this method of storing offers but small inspiration and aesthetic pleasure and thus cannot be of primary but merely of secondary importance for the curator of a fungous garden.

(Laboratory of General and Applied Microbiology of the University of Amsterdam).

SOME REMARKS ON MICROPHOTOGRAPHY

by

T. Y. KINGMA BOLTJES

(Received October 15, 1946).

In microbiology, bacteriology and other branches of science it is often not easy to make photographs of the objects to be studied, their structures lacking in contrast and being so fine, that they can hardly be resolved by our microscopes. To overcome these difficulties it is of importance to increase as much as possible the resolving power of the microscope, as well as the contrast in the objects. Now it is a well-known fact, that the resolving power can be improved by using light of the shortest possible wave-length. For the use of ultra-violet light with wave-length 275 $\mu\mu$ ZEISS manufactured quartz monochromatic objectives designed by KÖHLER and a quartz condenser and quartz oculars. This outfit, however, being rather expensive, is but rarely used. As nowadays the mercury vapour lamps, such as the Philips HP-300, are easy to handle, I thought it worth trying to apply the wave-length 365 $\mu\mu$, which may be easily realized by making use of a Schott UG 1 filter. Moreover the use of a mercury lamp has the advantage that it is very easy to get monochromatic light by making use of filters. After some experiments I found the following set of filters very satisfactory, each filter consisting of three glasscells 2 cm in diameter and having a capacity of 100 ml. The quantity of the different substances are given for 100 ml of solution.

As light of a wave-length of 365 $\mu\mu$ cannot be observed, there is, of course, the difficulty of focussing. KÖHLER in his quartz microscope used a special focussing ocular with a fluorescent layer. I tried a layer of impure anthracene, which gives a bright green light, and a layer of quinine sulfate. It was, however, impossible to focus by these means, the light being not strong enough. So I decided to make use of the constant difference existing between the adjustment with a visible wave-length and the wave-length of 365 $\mu\mu$. First I tried out whether the readings on the scale of

Wave-length 436 $\mu\mu$

I	2	mg rhodamine B
	2	mg eosin
	0,3	mg crystal violet
II	10	g neodymium nitrate
III	6	g cupric sulfate
	0.5	g methylene blue

Wave-length 546 $\mu\mu$

I	5	mg aurantia
II	10	g neodymium sulfate
III	6	g cupric sulfate
	1	mg malachite green
	1	mg methylene blue

Wave-length 577 $\mu\mu$

I	5	mg aurantia
II	50	mg eosin
III	6	g cupric sulfate
	0.5	g methylene blue

Wave-length 589-700 $\mu\mu$

I	5	mg aurantia
II	10	g neodymium nitrate
III	2	mg rhodamine B
	2	mg eosin
	0.3	mg crystal violet

the micrometer adjustment were constant when the image was well focussed. When taking care that the micrometer knob was always turned in the same direction in order to avoid back-lash this appeared to be the case. When the microscope is adjusted for λ 436 $\mu\mu$, the tube has to be raised to get the adjustment for λ 365 $\mu\mu$. So the focussing with λ 436 $\mu\mu$ has to be done by raising the tube. By means of six to eight test photographs the difference between the adjustment for λ 436 $\mu\mu$ and λ 365 $\mu\mu$ was measured. The change in the pictures tells whether the tube has to be raised or to be lowered. When the tube is slowly raised the picture becomes sharp at the border, then in the centre and finally the complete picture becomes unsharp. The difference in the adjustment between λ 436 $\mu\mu$ and λ 365 $\mu\mu$ has been determined for 4 Zeiss objectives in combination with a compensation ocular 20 $\times$. The camera was a Zeiss-Miflex, the length of the tube was 160 mm. The differences in adjustment between λ 436 $\mu\mu$ and λ 365 $\mu\mu$ were:

Apochromat	A 1.4	0.0016 mm
Apochromat	A 1.3	0.0012 mm
Fluorite	A 1.3	0.0024 mm
Achromat	A 1.25	0.0072 mm

The determination was carried out for 2 achromats, an old one and a recent one with iris diaphragm. In either case the difference was the same, so that it may be concluded that for both lenses the same construction is used.

When we now examine the pictures reproduced on plate I and those on the upper half of plate II, it is clear that notwithstanding

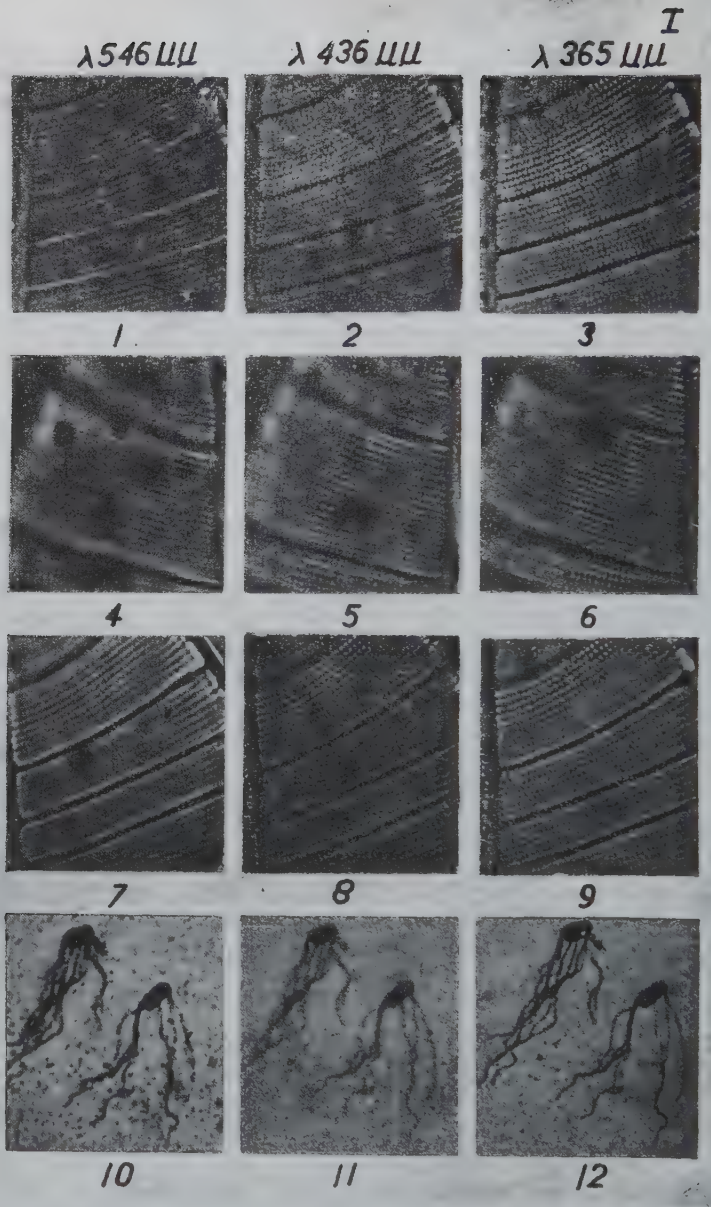

Plate I. Magnification 2500 ×, lightsource Philips HP-300.
Surirella gemma.

Fig. 1, 2 and 3 with fluorite 100 ×. A 1.3.
Fig. 4, 5 and 6 with apochromat 90 ×. A 1.4.
Fig. 7, 8 and 9 with achromat 90 ×. A 1.25.

Proteus mirabilis.

Fig. 10, 11 and 12 with fluorite 100 ×, A 1.3.

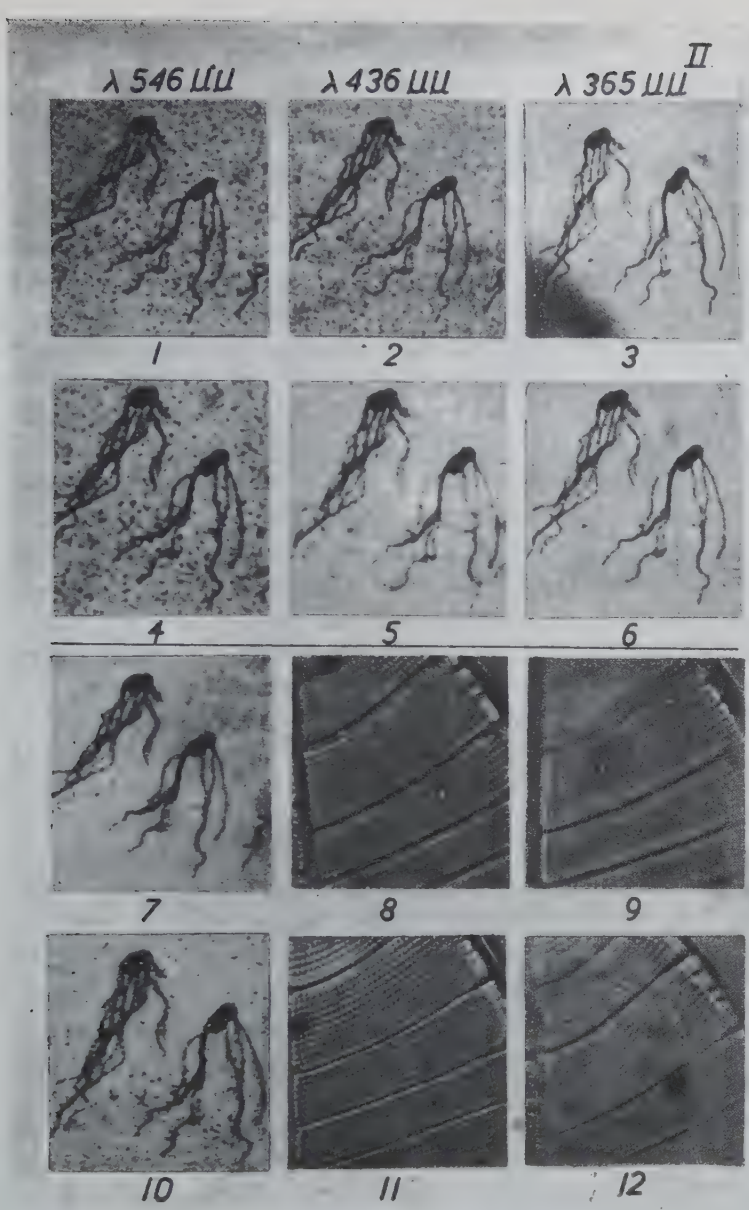

Plate II. Magnification 2500 ×, lightsource Philips HP-300.

Fig. 1, 2 and 3 with apochromat 90 ×. A 1.4.
Fig. 4, 5 and 6 with achromat 90 ×. A 1.25.
Fig. 7 light λ 365 μμ achromat 90 ×. A 0.73.
Fig. 8 light λ 365 μμ achromat 90 ×. A 0.73.
Fig. 9 light λ 546 μμ achromat 90 ×. A 1.25. A cond. 1.25.
Fig. 10 light λ 546 μμ achromat 90 ×. A 1.25. A cond. 1.25. A stop 0.6. Lightsource carbon arc lamp.
Fig. 11 light λ 475 μμ apochromat 90 ×. A 1.3.
Fig. 12 light λ 475 μμ achromat 90 ×. A 1.25.

the lenses are not corrected for the wave-length 365 $\mu\mu$, the use of this wave-length is a real improvement. If, however, by using this wave-length the contrast is diminished, we may expect that there will be no improvement. We further notice that there is no appreciable difference between the different objectives. This is in complete agreement with the statement of MELLON (8), who says that he is frequently surprised at the small difference existing in actual performance between good achromats and apochromats. This is not surprising, for in microphotography filters are often made use of so that the light used is more or less monochromatic. When the light used is not actually monochromatic, there will be a difference between an achromat and an apochromat, especially in the blue part of the spectrum. The difference is demonstrated by fig. 11 and 12 on plate II, where instead of the mercury lamp a coal arc lamp has been used and so the light was far less monochromatic. This could be improved by adding to the filters a glass cell with a solution of picric acid. The fluorite objective proved to be much better than the achromat, but not quite as good as the apochromat. As the mercury lamp gives a strong light and is more convenient in handling, it is superior to the coal arc lamp. The exposure time used for making the pictures with a 1000 $\times$ magnification varies between 5—10 sec. Even for the ultraviolet the exposure was between 5—20 sec. Preparations in canada balsam need twice or thrice as long an exposure as those in paraffin oil.

One of the reasons for making the latter experiments with more than one objective was the wish to obtain some pictures for demonstration to students, who had wanted to know whether apochromats are to be preferred to achromats and whether aplanatic or achromatic condensers are better than uncorrected condensers.

In my opinion the usual Zeiss achromats and fluorites are such good objectives that in microbiology or bacteriology there is hardly any reason to buy the more expensive apochromats. It even seems very problematic whether an apochromat A 1.4 is of any special use here. In fact the resolving power of this objective exceeds that of an objective A 1.3 in such a small measure that one can hardly expect to meet a case in which this slight improvement may be observed. From fig. 7 and 8 on plate II one gets an impression of the influence of the aperture. Here the aperture of the achromat A 1.25 was brought down to 0.73 by closing the diaphragm in the objective. By closing the diaphragm still further the structure of

the *Surirella* completely vanishes. As soon as the structure is resolved, the aperture has to be much larger in order to notice an improvement.

In pictures like fig. 7, plate II it is still more difficult to see any influence of the aperture. If here the aperture is made still smaller, the picture turns slightly more hazy.

In most textbooks there is usually paid little attention to the influence of the condenser on the quality of the microscopical image. In general it is suggested that in order to obtain the best results, the condenser should be either aplanatic or achromatic. See for instance MELLON (8), MICHEL (9), STONEY (13). However, I could not notice the least influence of the condenser on the quality of the photographic images, a fact which theoretically is supported by VAN CITTERT (3) as well as by ZERNIKE (14).

The advantage of the corrected condenser is that it allows the obtaining of a fairly good dark field by introducing a central stop in the diaphragm, whereas the darkfield of the uncorrected condenser is poor. Moreover it also gives a little more light, which may be of some advantage in microphotography.

A matter of interest is the influence of the condenser on the resolving power of the microscope. A theory for the forming of the microscopical image was given by RAYLEIGH (10), based on the theory of HELMHOLTZ (1874) and by ABBE (1). At first sight these theories do not seem to agree too well, so that they have often been the subject of discussions. On closer study, however, they are in complete agreement, as has been shown by ZERNIKE (14, 15) and others (5, 7).

The theory of RAYLEIGH is based on the fact that the image of a self-luminous point is not a point, but by the diffraction of light at the aperture of the objective becomes a disc of light surrounded by bright rings. They are often called AIRY discs, because AIRY (1834) was the first to solve the problem of the distribution of light in the image of a point source. From every point of the object a diffraction disc is formed in the image plane. The image of the object is the result of the co-operation of all these discs. When the object is self-luminous, so that every point may be considered as an independent source of light, the diffraction discs do not act one upon the other. When the discs overlap, the intensity of the image is the sum of the intensities of the overlapping discs. For the computation of the resolving power of the microscope RAYLEIGH considers two self-luminous points. For the resolving

power $\triangle$ he finds the equation $\triangle = \frac{\lambda}{1.64} A$ in which case the diffraction discs partly overlap. In practical microscopy, however, the object is not self luminous, but is illuminated and therefore not all the object points may be treated as independent sources of light. In this case the light coming from two points close to each other will be coherent, so that when the diffraction discs overlap, there will be interference and so instead of discs there will be one bright spot. This can be compared with the experiment of YOUNG in which two slits cut close to each other in a screen are illuminated. So we see that in case of a self-luminous object or completely non-coherent illumination the resolving power is better than with coherent illumination. This was already computed by RAYLEIGH. From a publication of VAN CITTERT (3) on coherence it can be deduced that the distance in which two points are to be considered as coherently illuminated becomes the smaller, the wider the opening of the illuminating beams is. Therefore the resolving power is increased by opening the diaphragm of the condenser. Two object points, separated by a distance that can just be resolved by an objective, become incoherently illuminated when the aperture of condenser and objective are the same. In case an oil immersion is used, oil should also be put between the condenser and the slide. The opening of the condenser, however, has the disadvantage that the general illumination becomes stronger and this, of course, will diminish the contrast between the two discs. This may prevent the seeing of the separation between the discs. We might obtain a better contrast if it would be possible to get coherent illumination with opposite phase instead of incoherent illumination. According to ZERNIKE (14, 15) this is more or less arrived at by illumination with a hollow cone of light, by introducing a stop in the centre of the diaphragm of the condenser.

The influence of the condenser on the resolving power is easier to be understood by means of the theory of ABBE. A fine grating was taken as object, the same as FRAUNHOFER used in his study of the diffraction of light. When a parallel beam of light is directed perpendicularly on a grating, part of the beam goes right through, whereas symmetrically on either side of this beam diffracted rays appear, which make different angles with the central beam. The angles of these diffracted beams increase along with a decrease in the period of the grating. The number of diffracted beams entering the objective depends on the period of the grating and the aperture

of the objective. By interference of these beams a number of bright discs are formed in the back focal plane of the objective, each of which is an image of the diaphragm of the condenser. The beams further on interfere in the image plane and an image of the object is formed.

An image is formed as soon as the central beam and one or two of the diffracted beams enter the objective. When only the central beam is caught in, of course no image is formed and the field is evenly lighted. In the theory of ABBE the aperture of the objective seems to have no other effect than the limiting of the number of diffracted beams, but this is not true, the objective as well having its diffraction effect.

According to ABBE the resolving power of the microscope is $\triangle = \frac{\lambda}{A}$, when parallel incident light is used. The resolving power can be doubled by making use of inclined beams, for then it is possible for the central beam and a single diffracted beam to enter the objective. The resolving power becomes $\frac{\lambda}{2A}$. In most cases, however, with inclined beams the quality of the image is poor and therefore usually all-sided oblique illumination is applied by opening the diaphragm of the condenser. In different textbooks (9, 11) it is suggested that in the latter case the resolving power is the same as in the case of the inclined illumination. VAN CITTERT (2, 4) already has found that this is not true. He noted that the ratio between the resolving power with parallel light and with all-sided oblique illumination is not 2 but 1.2.

This question has also been studied by GORTER and myself (6)

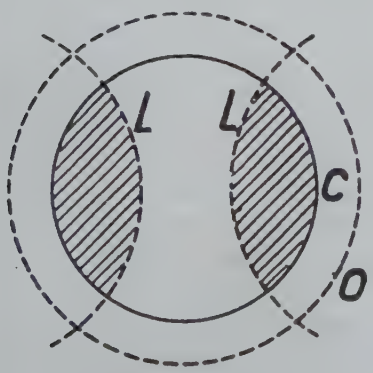

Fig. 1

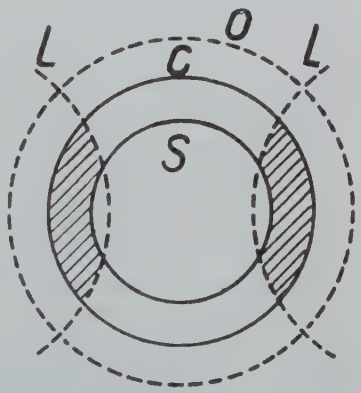

Fig. 2

from another point of view than that of VAN CITTERT (2). As has already been stated, in the back focal plane of the objective we see the images of the diaphragm of the condenser. In fig. 1 and 2 the dotted circle O is the aperture circle of the objective, whereas the drawn out circle C is the aperture circle of the condenser. The structure of the grating is so fine that in using parallel light no beams of the first order can enter the objective. By making use of the oblique rays from the condenser, part of the beams of the first order as well will enter the objective. To ascertain the amount of light of the beams of the first order the objective circle O is removed over the distance $\frac{\lambda}{d}$ (the dotted line L in figure 1 and 2 is a part of this circle). To get a picture of what we see in the back focal plane of the objective the condenser circle C should be removed over the distance $\frac{\lambda}{d}$.

Merely the amount of light in the hatched part can, by interference, form the image. The other part of the light only illuminates the field and diminishes the contrast of the image. The superfluous light can be eliminated by introducing a stop in the diaphragm of the condenser, as is shown in fig. 2. By these means the contrast will increase. The advantage of the stops in certain cases has been known since long and their function was explained by STONEY (13) and SIEDENTOPF (12). When the object has structures wider than $\frac{\lambda}{d}$, such stops should not be used.

When fig. 9 on plate II is compared with fig. 7 on plate I, we see the influence of the aperture of the condenser on the image. We see on fig. 9 that the structure is resolved somewhat better than on fig. 7, but that there is a little less contrast. The picture of fig. 10 plate II was taken after the introduction of a stop in the diaphragm of the condenser. When it is compared with fig. 4 on plate II, we see that, notwithstanding nearly the full aperture of the condenser was used, the contrast did not diminish.

In the phase contrast method of ZERNIKE it is also advantageous to diminish the central beam in order to increase the contrast and for this reason the small $\frac{1}{4}\lambda$ ring as well is made somewhat absorbing (5).

Theoretically GORTER could show that the visibility of an object depends on a, the ratio between the area of the hatched part of the condenser circle C and the area of the whole circle C. The

value of a depends on the nature of the object and on the observer. To test this theory a GRAYSON ruling with a grating period of 0.436 μ was observed with a ZEISS achromat with iris diaphragm. This diaphragm was calibrated with an ABBE apertometer. For different apertures of the condenser it was determined at which apertures of the objective the structure of the grating could just be noted. This was repeated after stops of different size had been introduced into the condenser. The a calculated from the data collected proved to be fairly well constant, *viz.* 0.15. So the developed theory was confirmed. The increase in resolving power with the condenser without central stop was in this case 1.6. This is more than VAN CITTERT (2) found, but this is of no importance, as the value of this factor depends on observer as well as on object. The same experiments were carried out with *Surirella gemma* in which case a was also fairly constant, *viz.*, 0.25. Here the increase in resolving power was 1.4. With *Amphipleura pellucida* mounted in realgar the gain in resolving power was 1.7. So we see that it is not possible to tell in which degree the resolving power will increase by making use of a condenser, but it is certainly much less than 2.

These experiments also allow of the conclusion that, an oil immersion being used, the application of the full opening of the condenser will increase the resolving power in a very small degree. Supposing that, by means of an oil immersion A 1.3 and light with a wavelength of 564 $\mu\mu$, we observe gratings which can be seen when $a = 0.25$. If the aperture of the condenser is 0.9, a grating with a period of 0.303 μ can just be resolved. By making the aperture of the condensor 1.3, this period will be only 0.290 μ. When we consider this and also bear in mind that usually the contrast becomes very weak by opening the condenser too far, so that it is often advised to make the aperture not larger than $^1/_3$—$^1/_2$ of that of the objective, it is clear, that the statement of MICHEL and REINERT, that usually the maximum aperture of the condenser does not need to exceed the figure 1, can be corroborated.

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(From the Laboratory of the „N.V. Nederlandsche Gist- en Spiritusfabriek", Delft).

THE ZBINDEN METHOD FOR THE MICRO-ESTIMATION OF COPPER AFTER PRELIMINARY DEPOSITION OF THE METAL BY ELECTROLYSIS

by

B. ELEMA

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Introduction.

Some years before the last war we needed a rapid routine method for the determination of micro quantities of copper in biological material. This method should be of such simple handling that it could be carried out by untrained personnel.

The wellknown colorimetric methods using the dithizon or carbamate reagents take too much time and need carefull attention of experienced workers to obtain reliable results.

First we tried to develop a volumetric micro-method using titanous chloride as a reducing agent. Though good results were obtained even in the presence of iron, this method is rather intricate and we did not succeed in adapting it sufficiently to our special requirements [1]).

Of course from the beginning our attention had been drawn to the possibility of estimating micro quantities of copper by electrolysis. After Pregl (1) had described this method, many others have used it. Hernler and Pfennigberger (2), for instance, who also published a paper on this subject, obtained very reliable results with quantities of copper from 50 μg upwards.

A serious drawback of Pregl's electrolytic method is however, that it involves the use of a micro balance. This has, of course, been realised by several workers. Some have avoided this difficulty by redissolving the copper that had been deposited on the cathode,

[1]) Titration at a high temperature and potentiometric control were necessary.

Methylene blue, though advocated by Monnier (3) for this purpose, cannot be used to indicate the reduction of iron and copper separately. We have not been able to find a redox dye better adapted for this purpose.

and thereafter estimating the redissolved copper by some colorimetric method or by other means. SCHOORL and BEGEMANN (4), for instance, developed a volumetric micromethod based on HAEHN's jodometric estimation of copper. In this case however the reagents must be of extreme purity and one gets the impression that a fair amount of experience in micro-analytical methods will be necessary to duplicate SCHOORL and BEGEMANN's excellent results.

By far the simplest method for the determination of electrolytically deposited copper has been introduced by ZBINDEN (5).

ZBINDEN redissolves the copper by reversal of the electric current, which is then kept at a fixed amperage until a sharp drop occurs, indicating that all the copper has been dissolved. The period of time expired is noted and the quantity of copper is calculated by application of FARADAY's law.

It is clear that if reliable results can be obtained in this way, this method is very well adapted for routine work. Moreover it has the advantage that when an apparatus with multiple electrode units has been constructed, several analysis may be carried out simultaneously.

Considering all this it seems surprising, that ZBINDEN's method has not drawn more general attention, especially since in recent years it has been recognised how important a function copper has in fundamental biological problems. Perhaps one of the reasons lies in the fact, that ZBINDEN's description of his method and apparatus is rather meagre.

For instance, no warning is given to the inexperienced, that the electrolysis of micro quantities of copper needs special attention to ensure quantitative deposition of the element on the cathode. It seems therefore worthwhile, to describe our experiences with the ZBINDEN method and publish in detail the apparatus that we have been using now for many years, with very satisfying results.

PRELIMINARY EXPERIMENTS.

In his first paper ZBINDEN (5) describes experiments in which known quantities of copper from 0.006 to 2.5 mg were deposited on a platinum cathode and thereafter dissolved by a constant opposing current. The amperage in these experiments varied from 0.8 to 10 mA. The quantity of copper was calculated applying the equation:

$$0.3294 \times t \times a = \text{micro grams of copper} \qquad (I)$$

where t = time in seconds
a = current in mA.

The shortest period of time measured was 10 seconds, the longest 18 minutes and 47 seconds. A mean error of at its maximum 3.3 % was found in a series of seven experiments using 6.36 μg of copper each.

We started our experiments in a similar way using a solution of 0.1 N sulfuric acid containing 200 μg of copper in the form of copper sulfate. Some sodium sulfate was added to increase conductivity. The total volume of the solution was $\pm$ 10 cm^3 and the electrolysis was carried out in a Pyrex test tube according to PREGL's directions while the solution was boiling slowly. Under these conditions the last traces of copper had disappeared from the solution in about half an hour, as was ascertained by a control reaction with carbamate reagent. Without interrupting the electrolysis the contents of the Pyrex tube were then cooled by immersion of the tube in cold water, and then the current was reversed as described by ZBINDEN. By means of a rheostat this dissolving current was maintained at a fixed amperage between 0.2 and 5 mA.

For indicating instrument we used a so-called „Mavometer" of the manufacture Gossen (Germany). This is a needle galvanometer provided with a mirror to facilitate readings. One scalemark indicates 0.04 mA.

The moment when the drop in the current occurred was noted with a stopwatch. This drop was most pronounced for the higher rates of current, but rather faint for currents under 1 mA. Calculation of the quantity of copper dissolved on the basis of equation (I) provided the results given in Table I.

It is clear from this table, that though reproducibility for a definite dissolving current is good, the quantity of copper found is consistently too low. The deficit increases when lowering the rate of the dissolving current *i.e.*, the longer period of time for dissolving is used. Obviously, at the same time that the copper is being dissolved by the electric current it is also slowly attacked by the sulfuric acid in the medium in a competing reaction. If this is true, an improvement of results can be expected using a lower acid concentration. Some experiments to prove this were made in such a way, that after the electrolysis had been completed and the solution had been cooled, the Pyrex tube was withdrawn and replaced by a second tube containing 0.01 N sulfuric acid

Table I.

Estimation of copper after ZBINDEN in 0.1 N H_2SO_4 containing some sodium sulfate.

Copper taken	Dissolving		Copper calculated	Copper deficit
	Current	Time		
μg	mA		μg	μg
200	5	1′57″	193	7
200	1	9′25″	186	14
200	1	9′21″	185	15
200	0.2	41′45″	165	35

Table II.

Estimation of copper after ZBINDEN in 0.01 N H_2SO_4 containing 0.25 % potassium sulfate.

Copper taken	Dissolving		Copper calculated	Copper deficit
	Current	Time		
μg	mA		μg	μg
198 [1])	5	1′58″	194	4
200	1	9′40″	191	9
200	0.2	43′45″	179	21

[1]) Some loss due to incomplete deposition of copper.

to which had been added 0.25 % potassium or sodium sulfate. During this manipulation, voltage was maintained permanently on the electrodes, which were washed with a stream of distilled water during the withdrawal of the first Pyrex tube. Redissolving of the copper by reversal of current was then carried out in the second tube. The results are given in Table II.

Comparing Tables I and II we see that especially for the lower dissolving currents *i.e.*, for longer dissolving times, the deficit in copper for the experiments in 0.01 N sulfuric acid is distinctly lower than in 0.1 N acid.

The supposition that copper after electro-deposition is slowly dissolved by the action of dilute sulfuric acid, was confirmed by immersing an electrode on which 10 μg of copper had been deposited, in 0.1 and in 0.01 N sulfuric acid containing 0.25 %

potassium sulfate. After a lapse of ten minutes visual inspection showed, that even the last trace of copper had disappeared from this electrode.

In one or two experiments, with a dissolving current of 1 mA, we tried the effect of omitting all acid. Curiously enough, the calculated copper values were not noticeably higher than those obtained in 0.01 N sulfuric acid. It was noted however, that during the dissolving process the thin layer of copper took on a greenish colour. Perhaps secondary reactions occurred in this case also but on the other hand it is possible, that our milliammeter indicated somewhat low. A similar experiment with a low dissolving current could perhaps have elucidated this point.

In any case it will be clear from the above, that with higher acidities and low rates of dissolving current the application of equation (I) may give much too low results. It is somewhat surprising, that in ZBINDEN's results no indication whatever of this phenomenon can be found. Perhaps the acidity in his experiments was lower than in ours, though mention is made of the addition of a „drop of sulfuric acid". It is striking however, that in his second paper (6) on the estimation of the copper content of milk, ZBINDEN chose high dissolving currents, so that the longest dissolving time in these determinations was only 5 seconds. In this way, of course, very little copper will be dissolved by the competing reaction with acid, but in our opinion this method has disadvantages, as too much depends on the rapidity of adjustment of the current and of the sensibility of the milliammeter. Moreover, a special instrument for the accurate measurement of the very short dissolving time will be necessary. ZBINDEN does not mention how this problem was solved in his experiments.

If on the contrary the dissolving of the copper is carried out at a medium amperage (1 mA), the influences of the sensibility of the milliammeter and the rapidity of adjustment of the current will be much smaller. In this case an ordinary stopwatch can be used for the measurement of the dissolving time. Nevertheless, for very small quantities of copper it is to be expected that corrections will have to be applied.

A disadvantage of dissolving at a medium rate of current is, however, that the dissolving time will be longer and therefore more copper will be dissolved by direct reaction with acid. Fortunately, for a current of 1 mA these quantities are still relatively small, as may be seen from Table II.

Notwithstanding this, it will be advisable to carry out the dissolving process under exactly defined conditions, and to determine the necessary systematic corrections by standardization against solutions containing known quantities of copper, for instance from 10 to 100 μg. In this way eventual errors of the milliammeter and of the stopwatch are accounted for at the same time.

Experiments similar to those already recorded in Table II were therefore carried out, in which the dissolving current was invariably adjusted at 1 mA.

In order to lessen the effect of back E.M.F. the rheostat used for adjusting the dissolving current was wholly turned in before the current was reversed. Immediately after this had been done, the rate of current was fixed to 1 mA by readjusting this rheostat.

The expiration of the time needed for the dissolving of the copper, was arbitrarily chosen to be the moment, when the needle of the milliammeter passed the 0.8 mA mark on the scale of this instrument.

The results obtained under these standardized conditions are summarized in Table III [1]).

Table III.

Corrections for the standardized ZBINDEN method for the estimation of copper.

Dissolving current 1 mA.

Copper taken	Number of experiments	Dissolving time for a current of 1 mA	Copper calculated	Correction	
μg			μg	μg	%
100	4	4′46″, 4′50″, 4′44″, 4′49″	94.2, 95.5, 93.5, 95.2	+ 5.4	+ 5.7
50	4	2′24″, 2′25″, 2′23″, 2′24″	47.4, 47.8, 47.1, 47.4	+ 2.6	+ 5.5
25	3	1′14″, 1′14″, 1′15″	24.4, 24.4, 24.7	+ 0.5	+ 2.0
17.5	1	52″	17.1	+ 0.4	+ 2.3
10	3	31″, 30″, 33″	10.2, 9.9, 10.9	— 0.3	— 3.0
5	2	16″, 16″	5.3, 5.3	— 0.3	— 6.0

[1]) A standardized solution of copper sulfate acidified with some acetic acid (7) was used for adding the prescribed amount of copper from a microburet.

We see from this table, that even for very small quantities of copper the reproducibility of the results is surprisingly good. The correction in μg that must be applied to the calculated values decreases with decreasing quantities of copper, which could be expected in view of the fact, that the shorter the dissolving time, the lesser copper will be attacked by reaction with acid.

It is striking, that for the smallest quantities of copper the correction, though in absolute measure it has become hardly noticeable, is negative. Obviously in these cases the correction is determined mainly by the fact that in the very first moments of the dissolving process the rate of the current is somewhat lower than the value to which it is adjusted afterwards by means of the rheostat.

It will be clear from the above, that if micro quantities of copper upwards of 10 μg have once been deposited in reasonably pure form on a platinum electrode, subsequent determination by a standardized ZBINDEN method may give results which are at least comparable in accuracy with those obtained by most other micro-methods.

It was of course of adventitious interest to know something about the electromotive force necessary to apply at the electrodes for dissolving the copper, using a current of 1 mA. An ordinary voltmeter could not be used for these measurements, as it had an unstabilizing influence on the dissolving current and made the drop in this current at the end of an experiment much less sharp. Later on we had the opportunity to repeat these measurements with the aid of a valve potentiometer of very high input resistance, and registered an E.M.F. of about 2.05 Volts.

At the moment that all the copper had been dissolved and the current dropped, this E.M.F rose sharply to 2.7 Volts. Obviously the discharge potential for hydrogen at platinum electrodes in 0.01 N sulfuric acid and 0.25 % sodium sulfate is rather high.

Taking into account the experiences recorded above, an assembly of six electrode units and an electric control-apparatus was designed, description of which will be given in the next section.

CONSTRUCTION OF APPARATUS.

a. Electrodes and electrode vessels.

Figure 1 shows two sets of an assembly of six units. Each pair of electrodes is mounted on a rectangular slide A of a laminated Bakelite material. These slides can move up and down between Bakelite strips B fastened on a common

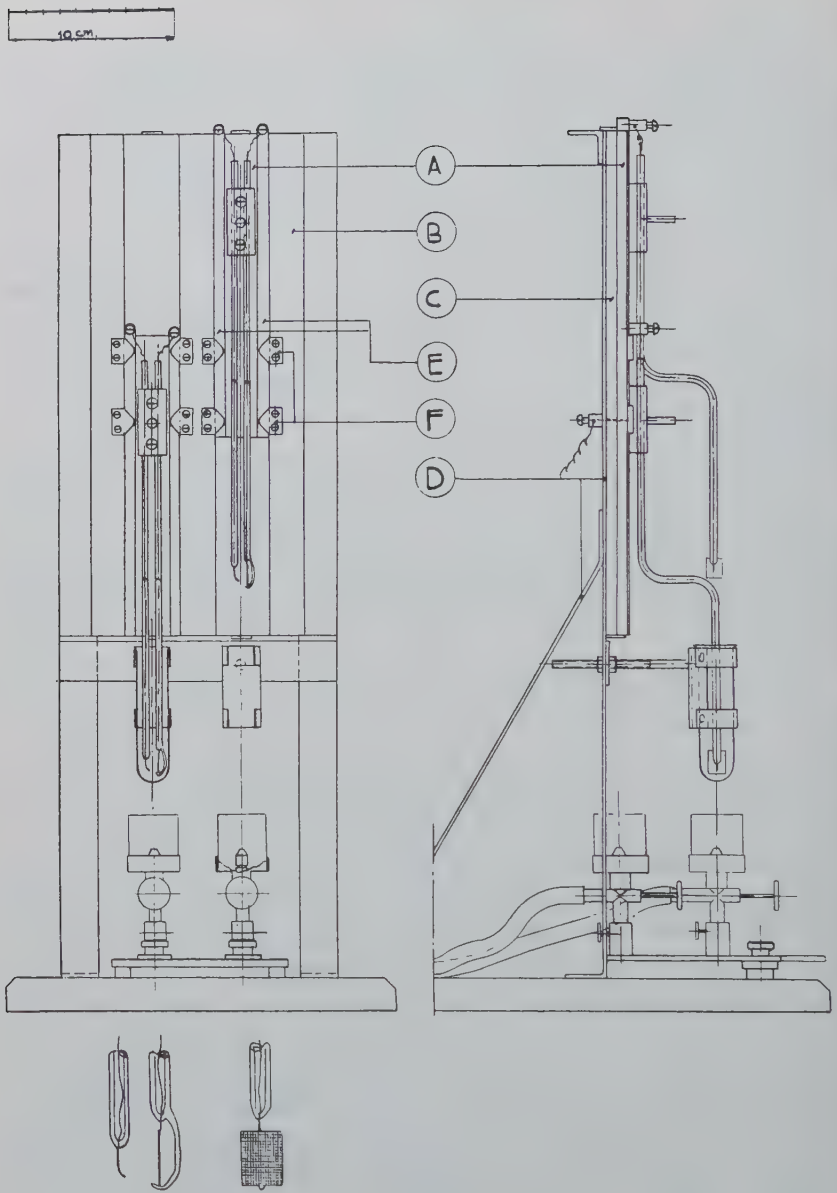

Fig. 1.

back panel C, which is mounted on a metal rack D. Stainless steel strips E, connected by binding posts and leads to the electrodes, are fitted along the electrode slides, which are held in place by the brass springs F. These springs are provided with tungsten contacts and terminate in binding posts behind the panel C.

The binding posts are connected by leads to the terminals of the apparatus controling the current for the electrolysis and for the dissolving process.

By drawing up the slide, the electrodes can be lifted out of the electrode vessel (a Pyrex test tube of 100 × 20 mm) without coming into contact with the walls of this vessel.

In Fig. 1 one of the two electrode units is shown in the high, the other unit in the low position. The electrodes proper are a platinum gauze cathode of 1 sq. cm. welded to a platinum wire of about 5 cm length and a platinum wire anode. Both wires are soldered to copper leads. The platinum wires are fused into glass tubes, for details c.f. Fig. 1. It is of course desirable that the six electrode sets are in every respect as equal as possible.

The construction of the micro burners used for boiling the solution during electrolysis and some further particulars can also be seen from Fig. 1.

b. Wiring diagram of control-apparatus.

The schematic wiring diagram of the apparatus which is used for controling the electrolysis and the dissolving process is shown in Fig. 2.

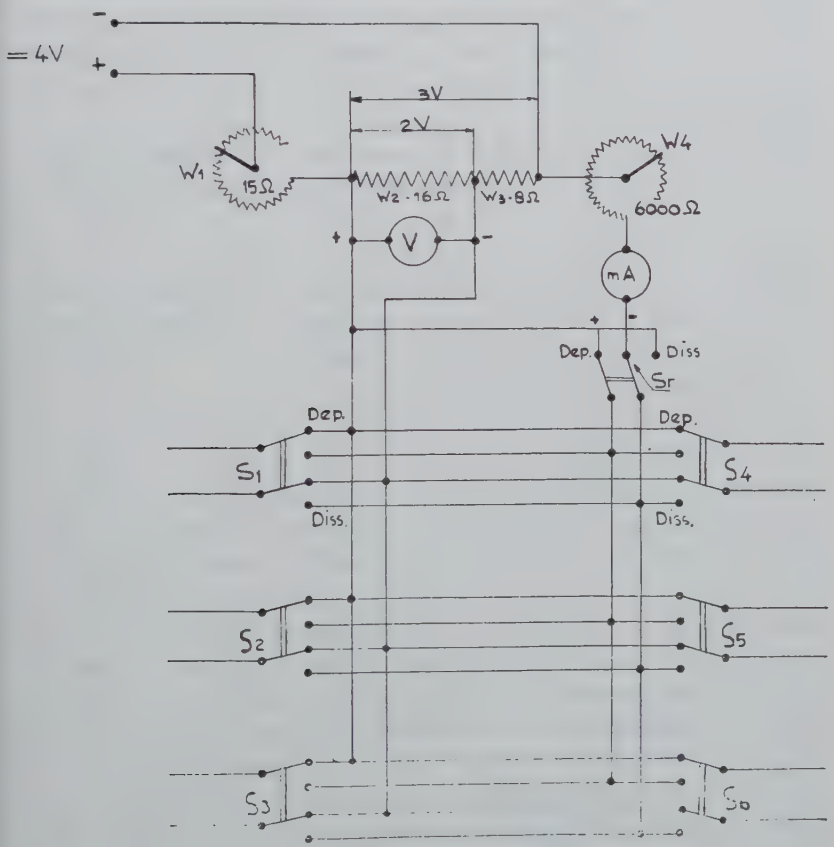

Fig. 2.

The circuit formed by the rheostat W_1 (15 Ω) and the fixed resistances W_2 (16 Ω) and W_3 (8 Ω) is fed by a 4 Volts lead accumulator. By means of W_1 and Voltmeter V the current in this circuit is adjusted in such a way, that the voltage drop along W_2 is 2 Volts, and consequently along $W_2 + W_3$ 3 Volts.

By means of the double pole throw switches $S_1 — S_6$ the electrolytic cells can be connected either with the 2 Volts or with the 3 Volts circuit. In the first case, the copper is deposited on the cathode; it is not possible with switches $S_1 — S_6$ in this position to reverse the current in the cells. If however one of the switches $S_1 — S_6$ is turned over, the current in this cell goes by way of the rheostat W_4 (6000 Ω), the milliammeter mA and doublepole throw switch S_7; by means of the lastnamed switch a reversal of the current can now be brought about.

From Fig. 2 it will be clear, that with this wiring diagram it is possible to reverse the current in one electrolytic cell, while the precipitation of copper in the other five cells still goes on.

As was already mentioned before, the milliammeter used was a „Mavometer" manufacture Gossen (Germany). Meters, switches and rheostats were mounted on a Bakelite panel and enclosed in an oak cabinet.

Suitable binding posts and leads were used for connecting with the binding posts on the back panel of the electrode assembly.

c. Standardizing.

A newly constructed assembly as has just been described will of course have to be standardized using solutions containing known quantities of copper as described on page 248. In our own case the results were practically identical with those given in Table III.

Estimation of copper in biological material.

a. Destruction of organic matter.

We obtained best results by the wet combustion method using sulfuric and nitric acid. For details of wet digestion methods c.f. (1, 8, 9).

The quantity of material to be taken, in general about 0.5–5 g dry substance, of course depends on the expected copper content. If a highly heterogenous material has to be analysed Pregl's (1) method of predigestion of a macro-sample with nitric acid may be used with advantage.

Digestion of a micro-sample was carried out in Kjeldahl flasks of 75 or 100 ml. First about 5 ml of concentrated H_2SO_4 of the highest purity is added and the flask is put aside for about half

an hour. In this way in most cases foaming and bumping during the destruction becomes much less troublesome. Then 5 ml of concentrated HNO_3 (sp.gr. 1.52) are added, the flask is placed on the Kjeldahl destruction rack and heating is begun cautiously. When the reaction has quieted down, more HNO_3 is added and the digestion continued until the solution has become water white in colour and no more redbrown fumes appear on addition of some HNO_3. The liquid is kept boiling untill SO_3 fumes are copiously evolved. Evaporation is then hastened by blowing a rather strong current of filtered air through the flask by means of a glass tube reaching almost down to the bulb. In this way the contents are evaporated to dryness. After cooling, the cake which has formed in the flask is brought into solution by boiling with about 5 ml of distilled water. The solution obtained in this way is practically free from nitric or nitrous acids and since the excess of sulfuric acid has also been driven off, the proper acidity for the electrolytic separation of copper can easily be established. This is done by adding 0.4 ml 25 % sulfuric acid and 0.5 g of pure potassium or sodium sulfate after the solution has been transferred from the Kjeldahl flask to an electrode cell, using about 4 ml of washwater.

Of course a blanc digestion using the same amounts of reagents is run at regular intervals and particularly whenever a new batch of one or more of these chemicals is taken into use. It is often necessary to purify the nitric acid by distilling it in an all-glass Pyrex distilling apparatus.

b. Electrolysis.

Using the apparatus already described on page 249, we have used PREGL's method *i.e.*, the electrolysis was carried out in the boiling solution at an E.M.F. of 2 Volts. This is easily accomplished by bringing all the switches (Fig. 2) in the positions „Dep" and adjusting rheostat W_1 till the Voltmeter V reads 2 Volts. Owing to the different construction of our electrodes (c.f. Fig. 1), we had to omit PREGL's cooler.

Evaporation of the solution is however compensated for by adding from time to time some distilled water, which is used at the same time to rinse down the sides of the electrode vessel.

For quantities of copper up to 200 μg an electrolysis of half an hour's duration proved to be sufficient [1]).

[1]) In doubtfull cases the liquid can very easily be submitted to a second electrolysis using a fresh pair of electrodes.

Under these conditions we have never observed any disturbing influence of zinc or iron. Occasionally however, a deposit of an abnormal gray brown character was obtained, which clearly contained considerable quantities of arsenic. In these cases the copper was purified by a second electrolysis as recommended by PREGL. This was done by redissolving the deposit in 0.1 N sulfuric acid and 0.25 % potassium sulfate by reversal of current, and thereafter carrying out the second electrolysis in this same solution. In a synthetic solution containing 100 μg of copper and 25 μg of arsenic the ZBINDEN method after the first electrolysis yielded 114 μg and after the second electrolysis 102 μg of copper.

On dissolving the impure copper deposit it was observed, that the drop in the dissolving current was much less sharp than usual. Probably this phenomenon may serve as an indication that a second electrolytic separation is advisable.

If greater quantities of zinc, iron or arsenic are present, it may be advantageous to carry out the electrolysis of copper in a medium containing some nitric acid as described by BENEDETTI–PICHLER(10) and others (2, 4) or even by other methods (2,11,12). In this case also complete removal of all superfluous acids used in the digestion is advantageous, so as to be able to adjust the solution to the desired composition.

If the electrolytic separation of copper in pure form has been brought about by one or the other of these methods, the subsequent estimation of the element by ZBINDEN's method has become a relatively easy matter.

c. Estimation by the standardized ZBINDEN method.

After the electrolysis at boiling temperature has lasted for half an hour, the solution is cooled by immerging the electrode vessel in a beaker containing cold water. Then the electrode vessel is withdrawn and replaced by a second Pyrex tube containing 10 ml of a solution 0.01 N in sulfuric acid and 0.25 % in sodium sulfate. During the withdrawal of the first Pyrex tube the electrodes are washed with distilled water.

Now one of the electrode switches S_1 — S_6 (c.f. Fig. 2) connecting the particular electrode pair is changed over into the position for dissolving and rheostat W_4 is turned fully in. Then switch S_7 is also turned to the „Diss." position and at the same time a stop-watch or other time marker is started.

Immediately afterwards the dissolving current is adjusted as quick as possible to 1 mA by means of the rheostat W and milliammeter mA. The needle of the milliammeter is constantly held under observation and, if need be, W_4 is readjusted.

The moment that all the copper has been dissolved becomes apparent, when the current decreases rapidly and cannot be kept constant any more. The watch is stopped at the moment that the needle of the milliammeter passes 0.8 mA. The time for dissolving is noted and the copper calculated using the equation:

Micrograms of copper $= t \times 0.3294$; t = time in seconds.

A correction must be applied which has been determined by standardizing apparatus and method against solutions containing known quantities of copper.

From this value finally the quantity of copper found in the blanc determination has to be deducted. Usually this blanc value is in the neighbourhood of only 1—2 μg of copper.

Summary.

ZBINDEN's method for estimating micro quantities of electrolytically separated copper by reversal of current has been investigated. Though the theoretical basis of the method proved to be more intricate than was to be expected from ZBINDEN's papers, standardization of the experimental conditions resulted in the development of a simple method for the determination of from 10—100 μg of copper. This method is very well adapted to routine work and may be carried out by untrained or hardly trained personnel.

Without doubt essentially the same procedure may be used for the estimation of greater quantities of copper. In that case standardization at a higher amperage of the dissolving current seems advisable.

Acknowledgment.

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(From the Baking-Department of the Nederlandsche Gist- en Spiritusfabriek, Delft).

THE CONTROL OF *BACILLUS VULGATUS* (FLÜGGE) MIGULA IN BREAD

by

S. BROEKHUIZEN

(Received October 3, 1946).

Introduction.

Rope is a bread disease, caused by *Bacillus vulgatus* (Flügge) Migula (up till now described in many publications (1,2) as *Bacillus esentericus vulgatus* Flügge).

This deformation of the bread — characterized by a typical and very unpleasant smell — is accompanied by a dampish, sticky bread crumb. The latter is the result of the decomposition of the starch by the bacteria. As a consequence the crumb texture disappears and the bread crumb can be drawn out into thin threads, which peculiarity gives the name to this bread disease.

The spores of *B. vulgatus* are exceptionally thermoresistent. This is the reason why spores, present in the flour, survive the baking process as the temperature in the bread crumb doesn't surpass 100° C. during baking.

It is a fact well-known to the bakers that rope develops easier in dark bread than in white bread in consequence of the fact that the bran contains more rope bacteria than the white flour from the inner part of the kernel. Now that the extraction rate of the flour has been raised to 85–90 % and therefore a great part of the bran has entered into the flour, the chance that rope develops in the bread has grown considerably. Moreover the development of these bacteria is promoted by warm and moisty conditions. In accordance with this, rope appears preferably where the newly baked bread cannot be cooled soon enough (for instance during the summer months; in narrow and insufficiently ventilated store-rooms).

As it is, however, the baker may take several precautions, by which the development of rope in bread can be avoided.

a) He has to keep the baking-house as clean as possible; no rests of dough and bread; no dirt.
b) The bread should be baked thoroughly; soggy crumb has to be avoided.
c) The bread has to be cooled as quickly and as thoroughly as possible.
d) Finally — and this is very important — the baker is able to add to the dough certain products by which the development of the bacteria is hampered.

Already in 1885 LAURENT (3) found that addition of acetic acid to the dough hampers the development of rope. Later on the salts of acetic acid (for instance calcium acetate) proved to have the same germicide value. Finally some other products are advised in preventing rope: calcium propionate, natrium benzoate and acid calcium phosphate.

The following investigation has been carried out with the purpose to find out in how far the preventatives mentioned give real satisfaction to the control of rope. Especially as to the action of acid calcium phosphate opinions differ.

FISHER and HALTON (4) obtained complete inhibition of rope by using 0.7 % acid calcium phosphate, while an amount of 0.4 % prevented rope development for 5–6 days.

KIRBY, ATKIN and FREY (5) prescribe so much acid calcium phosphate that the dough has a pH of 5.1.

O'LEARY and KRALOVEC (2) found calcium propionate to be two to three times as effective as calcium acid phosphate. They used an amount of 0.25–0.5 % calcium acid phosphate and 0.11–0.188 % calcium propionate.

Several investigators propagate the opinion that a pH of the bread of nearly 5.0 is essential for rope prevention. According to MORISON and COLLATZ (6) acetic acid, to SEIDEL (7) and WEIMERSHAUS and SVENSON (11) lactic acid, to HAVANTO (8) hydrochloric acid and to KIRBY, ATKIN and FREY (5) acid calcium phosphate should be added to the dough in such an amount that the hydrogen-ion concentration of the bread is brought to pH = 5.0.

With regard to acetic acid however it was already mentioned by KIRBY *et al.* (5) that it is not only the pH which hampers the development of the bacteria, but that it is a specific property of acetic acid. Several new data have been collected also in this respect.

Own investigations.

In all experiments the dough was made of „National Flour". This mixture is fixed by the government in accordance with the cereals available and milled to an extraction rate of 85½–87½ %. All doughs were made of two kg of flour from which 3 loaves of about 800 gram were obtained. Very soon after baking two slices of bread were removed from the middle-piece of two of the loaves by means of a desinfected knife and transferred into sterilized glass dishes. The glass dishes were incubated at 33° C. The development of rope was identified by the deterioration of the bread-crumb and by the appearance of the characteristic sickly smell.

In the experiments varying amounts of the following preservatives have been compared:

acetic acid	(concentration 80 %)
acetic acid	(„ 4.2 %)
lactic acid	(„ 50 %)
hydrochloric acid	(„ 36 %)
citric acid	(crystals)
calcium acetate	(patent Boehringer (9))
calcium propionate	
sodium benzoate	(patent Weimershaus and Hanke (10))
acid calcium phosphate	
acid sodium phosphate	

The data obtained in these experiments have been collected in Table I.

Another series of tests was made with the special purpose of comparing the preserving effect of acid calcium phosphate with that of acetic acid and calcium acetate. During these tests, symptoms of rope in the bread were checked with slices, stored in glass dishes at a temperature of 33° C. In addition to this the amount of spores of *B. vulgatus* in the bread was determined after storing them at a temperature of 37° C. for several days. In this determination the method according to Hoffman, Schweizer and Dalby (1) has been followed.

Two grams of the material are to be transferred aseptically to a 250 cc glass-stoppered bottle. About 10 grams of sea sand are added and then 96 cc of distilled water. From the obtained suspension several dilutions are made from 1 : 10 up to 1 : 100.000 by pipetting different amounts of the bread-suspension into culture tubes containing sterile broth. The broth has to be buffered on a

Table I.

Effect of varying amounts of different preservatives on the development of rope in bread.

Product	Amount in % of the flour	pH bread crumb	Bread volume in cc/kg flour	Rope after . . . days in thermostate at 33° C.					
				1	2	3	4	5	6
Acetic acid – 80 %	none	6.33	2680		+	+	+		+
	0.15	5.98	2775		—	?	+		+
	0.31	5.69	2830		—	—	—		—
	0.47	5.52	2885		—	—	—		—
Acetic acid – 4,2 %	none	6.12	2870		+	+		+	
	2	5.95	3015		?	+		+	
	4	5.80	3170		—	—		—	
	6	5.43	3070		—	—		—	
Lactic acid – 50 %	none	6.20	2885	+	+	+	+	+	
	0.3	5.88	2925		+		+		
	0.6	5.66	3045		+		+		
	0.9	5.50	3025		—		—		
Hydrochloric acid – 36 %	none	6.29	2840	+		+			
	0.3	5.98	2980	+		+			
	0.6	5.54	2945	—		+			
	0.9	5.13	2870	—		?			
Citric acid	0.3	5.55	2920	+		+			
	0.6	5.26	3020	—		+			
Calcium acetate	none	6.44	2825		+			+	
	0.36	6.14	2875		—			+	
	0.75	5.79	2945		—			—	
Calcium propionate	none	6.44	2825		+			+	
	0.36	6.11	2835		—			—	
Sodium benzoate	none	6.44	2825		+			+	
	0.15	6.46	2810		—			+	
Acid calcium phosphate	1	5.47	3125		—			+	
	2	5.07	3145		—			?	
	3	4.95	3115		—			—	
Acid sodium phosphate	0.5	6.02	2830	+		+			
	1	5.92	2900	+		+			

pH = 6.0. The tubes of nutrient broth are heated in a steam sterilizer for 30 minutes at 100° C.

Then the tubes are incubated for 48 hours at 37½° C. and examined at the end of this period for the presence of surface growth. Presence of a pellicle or any surface growth constitutes a presumptive test for the presence of rope producing spores (see photo). The number of rope producing spores per gram of bread is taken as the reciprocal of the highest dilution giving positive results.

Fig. 1. Series of test tubes showing the development of a pellicle on the dilutions 1 : 10 up to 1 : 100; the next dilution (1 : 150) shows no surface growth. Conclusion: ca. 100 spores per g of bread.

The results of the tests made according to the described method are summarized in the tables II, III en IV. In addition to the results of the tests for the amount of spores in the bread also the check in duplo on symptoms of rope in slices, stored at a temperature of 33° C. in glass dishes, are quoted in these tables.

Discussion of the results.

In considering the results of these tests, obviously not all rope-preservatives recommended in literature are capable of preventing entirely the development of „rope" in the bread. This is the case

Table II.

The influence of varying amounts of acetic acid (conc. 4.2 %) on the development of rope and rope spores in bread.

Amount of acetic acid	0		2 %		4 %		6 %	
	rope	spores per g of bread	rope	spores per g of bread	rope	spores per g of bread	rope	spores per g of bread
fresh	— —	none	— —	30	— —	60	— —	40
incubation time:								
1 day	— —	none	— —	none	— —	none	— —	none
2 days	+ +	> 100.000	— —	5.000	— —	20	— —	30
3 ,, ...	+ +	> 100.000	+ +	> 100.000	— —	10	— —	10
4 ,, ...	++ ++	> 100.000	+ +	> 100.000	— —	60	— —	20
5 ,, ...	++ ++	25.000	++ ++	25.000	— —	2.500	— —	none
8 ,, ...	++ ++	50.000	++ ++	> 100.000	+ +	25.000	— —	none
pH bread-crumb (fresh) . .	6.00		5.77		5.58		5.32	
Bread volume in cc per kg flour . . .	4500		4955		5050		5090	

Table III.

The influence of varying amounts of calcium acetate on the development of rope and rope spores in bread.

Amount of Ca. acetate	0		0.2 %		0.3 %		0.4 %	
	rope	spores per g of bread	rope	spores per g of bread	rope	spores per g of bread	rope	spores per g of bread
fresh	— —	250	— —	100	— —	40	— —	10
incubation time:								
1 day	+ —	20	— —	10	— —	none	— —	10
2 days	+ +	> 100.000	— —	none	— —	20	— —	none
3 „	++ ++	> 100.000	+ +	> 100.000	— —	500	— —	100
4 „	++ ++	> 100.000	+ +	> 100.000	— —	60	— —	40
5 „	++ ++	> 100.000	++ ++	> 100.000	— —	none	— —	10
pH bread-crumb (fresh) . .	5.70		5.52		5.45		5.60	
Bread volume in cc per kg flour . . .	4225		4260		4345		4430	

Table IV.

The influence of varying amounts of acid calcium phosphate on the development of rope and rope spores in bread.

Amount of acid calcium phosphate	0		0.25 %		0.5 %		1.0 %		1.5 %	
	rope	spores per g of bread	rope	spores per g of bread	rope	spores per g of bread	rope	spores per g of bread	rope	spores per g of bread
fresh	—	40	—	?	—	30	—	60	—	100
incubation time:										
1 day	—	none	—	100	—	none	—	none	—	10
2 days	—	20.000	—	1.000	—	500	—	none	—	none
3 „	+	100.000	+	5.000	+	10.000	+	500	—	20
4 „	+	100.000	+	100.000	+	100.000	+	50.000	+	80
5 „	+	100.000	+	100.000	+	100.000	+	100.000	+	50.000
pH bread-crumb (fresh)	5.88				5.52		5.28		5.12	
Bread volume in cc per kg of flour	4730		4740		4695		4475		3685	

for hydrochloric acid, citric acid and also for lactic acid. The amount of lactic acid needed to prevent rope makes the bread inedible, because of its sour taste.

Conclusive results of all examined preservatives were obtained only after acetic acid, calcium acetate and calcium propionate had been added to the dough.

From the tables II and III it follows conclusively, that a complete checking of the development of *B. vulgatus* in the bread is possible by adding 4 % household-vinegar or 0.3 % calcium acetate to the dough.

From table IV it can be concluded, that acid calcium phosphate does not fully prevent the development of rope. Even at the highest amount used (1 %), which made the bread too sour, the occurrence of rope could be clearly stated after 4 days. It only can be said, that an addition of at least 0.5 % of acid calcium phosphate sufficiently protects the bread against rope for practical purposes under normal conditions for about two or three days. After storing the bread for more than 3 days acid calcium phosphate doesn't protect it any more.

The test according to HOFFMAN *et al.* (1) reveals the striking fact, that mostly after a storage of 1 day no spores were to be found in the bread. When the test data presented are studied more closely, it appears — as might have been expected — that in freshly baked bread spores of rope are to be found. Under favorable conditions after 1 day these have developed into bacteria, though these bacteria apparently have not yet reached the sporeforming stadium. On the third day spores are to be found in the breadcrumb again.

It follows from the tests, that spores of rope are neither able to withstand an addition of at least 6 % household-vinegar or 0.3 % calcium acetate, so that finally no spores are to be found in the bread.

According to the test-results this is obviously not the case for acid calcium phosphate.

Summary.

The object of the investigations was to obtain an impression of the effect of several preservatives on the development of *Bacillus vulgatus*, which causes „rope" in bread. Special attention was paid to the value of acid calcium phosphate, which is considered in

current literature as an outstanding rope-preservative. This opinion was not confirmed by experimental investigation. On the contrary, it had to be stated that an addition, which caused the pH of the bread-crumb to decrease to the amount of 5.1, gave no definite protection against rope. Even if the opinion, that in this way the bread could be protected sufficiently for practical purposes, would hold, the drawbacks of a somewhat sour taste, a small volume of the bread and a rather expensive product remain to be considered.

It can be clearly seen from the results of the investigation, that acetic acid and calcium acetate check the development of *B. vulgatus* completely. Both products have moreover the practical advantage of being cheap.

Acknowledgement.

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MICROBIOLOGICAL EXPERIENCES IN JAPANESE CAMPS FOR PRISONERS OF WAR

by

G. GIESBERGER

(Received October 31, 1946).

> Bacteriology has not merely unveiled to the human mind unknown and unconjectured aspects of the microscopical world, it has moreover furnished means for the mastering of the benefits this microscopical world can offer and for the controlling of the misery which it can cause. It has tremendously extended our power over the phenomena of life.
>
> M. W. BEIJERINCK. Inaugural address, Delft. Sept. 26th 1895.

The enthusiastic words in which the great microbiologist BEIJERINCK tried to reveal the importance of his branch of science have, if ever, found their response in the conditions occurring in the numerous Japanese camps for prisoners of war and for internees during the occupation. Against the great misery caused by various infectious diseases, which through insufficient means were hard to combate, many cases exist, where the inhabitants of the camps were glad to make use of the special potencies of various micro-organisms for the improvement of conditions they were living in.

My endeavours in the microbiological field in the camps already date from the first months. In order to surmount the difficulties met with in the baking of bread with the available sour dough, it was tried to isolate a yeast species, which might be cultivated on a large scale for the baking. The isolation actually succeeded by streaking the sour dough on a slice of a ripe papaja, as nutrient agar was lacking. A flat well-closed tin box served as petri dish. The yeast species present in the sour dough thrived so well on the slice of papaja, that after some transfers a pure culture was obtained. As the camp was abolished shortly after and the next one was provided with baker's yeast by the Japanese, a further development on a large scale has not been realized.

New and strong demands for applied microbiology in the new

camp (4th and 9th Depot-Batallion at Tjimahi) came from medical quarters. Many inhabitants suffered from a more or less continuous diarrhoea, due to gastroenteritic disturbances caused by the abnormal nourishment. Several physicians applied chalk as an astringent, which, however, was only available in very small quantities. As lime stone was found by chance in large quantities in this camp and when burnt produced a lime of good quality, it was resolved to prepare precipitated chalk from this lime. For the conversion of $Ca(OH)_2$ into $CaCO_3$, carbon dioxide was used which was produced by a microbiological process.

The remains of rice from the kitchen, which in that period were still available in sufficient amount, served as raw material. Later, when hardly any rice was available, the remains of bread were used successfully. The rice was subjected to a process of saccharification by means of the fungus *Rhizopus oryzae*, occurring in „ragi cakes". The thus obtained sweetish tasting paste (known as „tapé" to the native population) after having been mixed with water was left to a spontaneous fermentation in iron tanks of $\pm$ 100 l (derived from disused kitchen wagons). Yeasts as well as lactic acid bacteria took part in this fermentation process. The fermentation of a single lot took about two days. By means of this fermentation process during many months a continuous, quite satisfactory production of carbon dioxide was arrived at (not rarely over 1 l per minute). In this way chalk of good quality was obtained. It not merely served medical ends but as well was used as the raw material for a tooth paste prepared in the camp. During nearly one year, the period in which this branch of industry worked, some hundreds of kilograms of dried chalk and several thousand portions of tooth paste have been produced.

As the whole system was based on the microbiological conversion of rice amylum by *Rhizopus oryzae* (comparable with the so-called amylo process of the breweries) and as in many cases the preparation of food yeast was based upon it, I shall summarily mention some questions bearing on this process. Initially the rice has been inoculated with finely ground ragi-cakes, which were obtained from outside the camp. The native population prepare these cakes out of dough from rice flour in which the fungus develops spontaneously and forms spores. In dried condition the cakes contain the fungus so to say in conserved condition. Later spore suspensions have served as inoculum of the fungus. These were obtained by storing a moistened empty rice bag, which will always contain some rests

of rice, under moist conditions. After a few days a thick fungus growth forms on the bag, first of a dirty white, but soon of a blueish black due to the numerous spores. These spores appeared ideal for the induction of a rapid and constant saccharification of the rice. The condition of the steamed rice appeared of major importance for the succeeding of the saccharification process. In fact when the rice is too moist and sticky, the process may fail completely. Instead of the favourable fungus, bacteria and yeasts develop, inducing a rapid rise in temperature and finally the rice turns into a disagreeable, musty smelling, slimy mass. Good aeration and not too high a temperature (of course no self-heating may occur) are essential. Especially when the remains of rice from the kitchen, which were often strongly contaminated, had been put to use, difficulties were often met with. When, however, freshly steamed rice from the kitchen had been used, the process took nearly always a favourable course. In 2—3 days the saccharification had proceeded sufficiently for the complete conversion of amylum into dextrin, maltose and glucose. A sweetish liquid dripped from the sticky mass, which after thickening by evaporation could furnish a native tit-bit, the „brem".

Of much greater importance, however, than this microbiological source of chalk, was the production of a „food yeast". Numerous inhabitants of the camp, as a result of the inappropriate nourishment suffered more or less seriously from Vitamin-B deficiency. As in that period baker's yeast could be had at a not too exorbitant high price from outside the camp in Tjimahi, cheap raw material was needed would it be economically allowed to culture one's own yeast. Again rice (later bread) remains of the kitchen were appropriate for this use. Two methods existed for the putting to use of this material after saccharification by *Rhizopus oryzae*. The first one consisted in preparing a wort from the saccharified rice by diluting with water, inoculating with yeast and, when the fermentation had come to a close, providing the patients with this wort as a whole. This system might be claimed as the most suitable under conditions such as they existed in most of the camps. In fact in Ambon, Batavia and Singapore etc. this method has been followed starting from fresh rice from the kitchen. It has been tried, prior to the inoculation with the yeast, to sterilize the wort as far as possible; by lack of fuel, however, this could not always be realized.

In Tjimahi, however, the remains of rice to be worked with

were often contaminated in such a measure, that consumption of a yeast containing wort, prepared from such material was hardly tempting. Another method in this case led to a satisfactory solution. In fact it appeared that after fermentation of the wort from tapé, such as this took place in the CO_2 tanks for the production of chalk, on the surface of the fermented wort left in the air a definite yeast species developed spontaneously and abundantly. This yeast, (perhaps a *Torula* species, as in the literature on yeasts in tapé a species of *Torula* is mentioned as normally occurring) appeared to find favourable growth conditions in the oxidising of alcohol, lactic acid etc. occurring in this medium. In order to favour the development later some dedek (bran from the rice mills mixed with the pericarps) has been added to the medium. By simple means the yeast could be obtained in fairly pure condition by skimming the thick, rimpled pellicle off the surface followed by a further purification by means of repeated washings. In this way a thick yeast somewhat smelling of cheese was obtained which in consistency did not differ much from baker's yeast. The yield was very high, the more so after a special race with large cells and high production had been selected. From 100 l steamed rice about 15 kg of yeast could be obtained. When by means of control tests by a number of persons it had been certified that the yeast had about the same value as pressed yeast in combating the phenomena of Vitamin B deficiency, it was resolved to cultivate this yeast on as large a scale as possible. Thus fermentation basins with a large surface were constructed in which the tapé wort was poured in a thin layer of 10—15 cm. A shed with a cement floor served very well for the instalment of the fermentation basins, their walls consisting of wooden boards. These boards were first cemented on ridges in the floor. Later, when cement was lacking the boards were made leak proof by plastering them on the outer surface with clay from the rice fields. When this industry was at its full height the total surface of the yeast tanks came up to 75 m^2. The layer of the yeast in the basin was daily skimmed off, leaving merely a thin film and after 24 hours the yeast had again developed into a thick layer. From each basin yeast could be harvested during 7—10 days, depending on the degree of saccharification of the rice. Many hundreds of inhabitants of the camp were thus provided with a fair quantum of yeast, in pasteurised condition, supplied with sugar and cinnamon for the improvement of the taste. A part of the residual fairly acid wort from the yeast tanks was finally

used in the manufacturing of paper for the breaking up of the fibres of the waste paper during the boiling.

Leaving the cultivation of yeast I will further point to the fact that in many camps alcohol has been produced for medical ends (secretly also for consumption) by means of fermentations of solutions containing sugar. The destillation of alcohol through lack of material was often realized by means of wondrously improvised destillation apparatus. In Tjimahi for instance I made use of a large enamelled teakettle as a still, the spout being connected with the cooler which next to a water jacket out of tin contained as essential part the glass stringtube of a well-known violinist.

In Tjimahi the thus obtained alcohol was moreover used for the preparation of vinegar for use in the kitchens as soon as this payed, the vinegar which could be obtained from outside the camp being high in prize and very low in quality. The alcohol, however, was not conducted over chips of wood but over long strips of bag cloth strained parallel one to the other in a wooden chimney. When after some lapse of time a flora of acetic acid bacteria had developed (among which *Acetobacter xylinum* occurred regularly), the conversion of alcohol into acetic acid took a rapid course. The correct moment for bringing the process to a stop was determined titrimetrically. In this connection it may be observed that in the absence of any indicators, natural indicators had to be looked for. A red substance in the marrow of the root of the so-called kajoe setjang appeared to serve this end successfully, while later a dark red substance occurring in the root of a species of mangrove was appropriate as well.

As a final example of the application of microbiology the preparation of „tempé" from soybeans may be cited. Experience learns that soybeans as such are hard to digest. The native population have always prepared a much valued, tasty and nourishing product from soybeans, the „tempé kedelee" by letting boiled or steamed beans grow mouldy. In many cases it is *Rhizopus oryzae* or a nearly related fungus which plays its parts here and which by its development induces a sufficient breaking up and thus a better digestibility. It stands to reason that the preparation of tempé was taken up in the camps in as far as soybeans were available. Difficulties have been often met with, however, because the desired fungus did not or insufficiently develop and putrefying bacteria took the leading instead. By slightly acidifying the soybeans, the development of the bacteria was inhibited, but the

actual factors benificent for moulding consisted in a good aeration and the maintaining of a not too high temperature. Care had to be taken that the bean mass was not subjected to the moulding process in too moist a condition. After this experimental evidence in most of the camps the production of a tempé of sufficient quality was arrived at and thus the inhabitants of the camp could enjoy the benifit of this much valued food stuff.

I have now reached the end of this survey of the principal applications of microbiology in the Japanese camps for prisoners of war. If most of the camps had not been removed over and over again, doubtless much more might have been accomplished in this field. So in Tjimahi plans existed in an advanced state for the preparation of ammonia from urine by means of urea bacteria in order to produce a lye for the manufacturing of soap. When, however, the total sum is considered there is in my opinion every reason for satisfaction with the results obtained, which without the widened field of view which applied microbiology offers us, would never have been realized.

(Laboratory of the „N.V. Nederlandsche Gist- en Spiritusfabriek", Delft).

SACCHAROMYCES MARXIANUS HANSEN

by

J. LODDER

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I. Introduction.

A casual comparison made between the description which Stelling–Dekker (6) gave of *Zygosaccharomyces Marxianus* (Hansen) Guilliermond et Negroni and that which the authors (1) gave of *Saccharomyces macedoniensis* Diddens et Lodder showed a striking agreement between both. This observation led to a somewhat closer study of these two yeasts.

A short survey of the origin of various strains of the two yeasts, mentioned in literature, and their classification follows:

The original description of *Zygosaccharomyces Marxianus* was given by Hansen (3) in 1888. He studied a strain, isolated by Marx from grapes, which he classified in the genus *Saccharomyces* as *Saccharomyces Marxianus*.

In 1929 Guilliermond and Negroni (2) gave a more detailed description of this yeast. As they had observed that the asci result from a conjugation of two cells, they transferred this species to the genus *Zygosaccharomyces*. It is, however, doubtful whether the French investigators studied a culture of the authentic strain of Hansen.

In 1931 Stelling–Dekker who studied a culture of the French strain, did agree with the classification of this yeast as a *Zygosaccharomyces*. She, however, did not consider *Zygosaccharomyces* and *Saccharomyces* as different genera, but as subgenera of one genus: *Saccharomyces*.

In 1938 Sacchetti (5) described a strain of *Zygosaccharomyces Marxianus* isolated by him from sewage of a sugarfactory.

The five strains of *Saccharomyces macedoniensis* on which the description of this species is based, came all from Castellani. Probably they had been isolated from sputum or from the air.

For comparison the descriptions of *Zygosaccharomyces Marxianus*

and that of *Saccharomyces macedoniensis*, as given by the various authors follow:

	Zygosaccharomyces Marxianus	*Saccharomyces macedoniensis*
Shape and seize of the cells grown in malt-extract for 24 hours	Cells are oval or elongated (2.5–5) × (6–13) μ, single or in pairs (STELLING–DEKKER). (3–6) × (6–12) μ (SACCHETTI).	Cells are oval, (3,5–5) × (5–8) μ, or elongated 4.5 × 12 μ; cells are single or in pairs.
Growth in malt-extract after a longer incubation time	Mouldlike flocks in the liquid. After 2 or 3 months a thin pellicle is formed (HANSEN). A heavy ring, a sediment and a thin pellicle are formed after 1 month (STELLING-DEKKER).	After 6 days a sediment and a ring are formed.
Growth on maltagar	After 8 days cells are oval or elongated, (2–5) × (6–33) μ, sometimes in chains. On streak-culture growth is grey, a little tough, shining, with a netlike structure, margin sinuous (STELLING–DEKKER). On streak-culture growth is greyish with some transverse structure, margin scalloped (SACCHETTI).	After 6 days cells are oval or elongated, (2–5) × (5–19) μ. Cells single or in pairs. Streak-culture is yellowish-white, smooth, soft and shining.
Ascopores	Ascopores are reniform, round or oval, smooth, average diam. 3.5 μ. HANSEN did not make mention of conjugation preceding ascospore formation; the other authors stated that the ascospores were formed after isogamic or heterogamic conjugation or parthenogenetically.	Ascopores are oval or reniform; 4 or less ascospores per ascus. No conjugation precedes ascospore formation.
Ability to ferment various sugars	Glucose, fructose, mannose + Galactose +	Glucose, fructose, mannose + Galactose +

	Zygosaccharomyces Marxianus	*Saccharomyces macedoniensis*
	Saccharose +	Saccharose +
	Maltose —	Maltose —
	Lactose —	Lactose —
	Raffinose + $^1/_3$	Raffinose + $^1/_3$
	Inulin +	Inulin +
Nitrate assimilation	Negative	Negative
Growth with ethylalcohol as sole source of carbon	Growth (STELLING-DEKKER)	No growth
	Growth very doubtful (SACCHETTI)	
Maximum temperature	Nearly up to 45° C. still growth	At 41° C. good growth
Production of fruit ether odour	Not mentioned	A slight odour of fruit ether develops.

As is evident from a comparison between the above mentioned descriptions there exists a very great resemblance between both species. A conjugation which precedes ascospore formation and generally occurs in *Zygosaccharomyces Marxianus*, but never has been observed in *Saccharomyces macedoniensis* is the only important difference. Consequently one has the impression, that these two yeasts, which were classified in different genera, do not even belong to different species, but more likely belong to one species which occurs in different forms.

The investigations of WINGE and LAUSTSEN (7) and later those of LINDEGREN and LINDEGREN (4) have shown, that there does not exist a real difference between the genera *Saccharomyces* and *Zygosaccharomyces*. The *Saccharomyces* species, indeed, have vegetative cells which are diploid, whereas in the genus *Zygosaccharomyces* the vegetative cells are generally haploid. WINGE and LAUSTSEN, however, often succeeded in bringing yeasts with haploïd cells into the diplophase and those with diploid cells into the haplophase. So, *e.g.*, they isolated one ascospore from an ascus of the Danish baking yeast, a yeast with diploid vegetative cells belonging to the species *Saccharomyces cerevisiae*. The culture grown from this ascospore remained haploid till it was transferred to a special medium, stimulating ascospore formation — slice of carrot, gypsum block —. The cells conjugated on both media; on

gypsum even ascospores developed in the zygotes. So, a culture grown from an ascospore of a yeast belonging to the genus *Saccharomyces* fully behaved as a *Zygosaccharomyces*.

The Danish investigators further succeeded in obtaining a hybrid by mating a *Saccharomyces* species — *S. cerevisiae*, Rasse XII — with a *Zygosaccharomyces* species — *Z. Priorianus* —. Herefrom it is obvious, that there probably exists a closer relation between the parents of this hybrid than is expressed by classifying them in different genera.

According to WINGE and LAUSTSEN and to LINDEGREN and LINDEGREN it is very doubtful whether the genus *Zygosaccharomyces* ought to be maintained.

In this connection it is obvious to suppose that *S. macedoniensis* with diploid vegetative cells is the diplophase, and *Z. Marxianus* generally with haploid vegetative cells represents the haplophase of one species. When a culture of *Z. Marxianus* could be brought into the diplophase and one of *S. macedoniensis* into the haplophase it would have been proved that this supposition is true. In this case, moreover, it would have been emphasized once more that the genus *Zygosaccharomyces* is no longer valid.

II. EXPERIMENTS.

1. *Zygosaccharomyces Marxianus.*

In the yeastcollection of the „Centraalbureau voor Schimmelcultures" at Delft, two strains of *Z. Marxianus* are present *viz.*, the strain studied by STELLING-DEKKER and the strain of SACCHETTI.

The first strain has been in the collection since 1922. When I studied this strain in 1940 a dissociation (mutation?) had appeared. The strain consisted namely of a mixture of two cultures: one developed curled colonies on maltagar or on maltgelatine, whereas the other had smooth colonies on these media. When both cultures had been kept for some time on maltagar separatively and were then cultivated on maltgelatine the curled culture generally developed curled colonies, but also some smooth ones; the smooth culture besides smooth colonies also developed some curled ones. So the dissociation was reversible. The curled culture had lost the ability to form ascospores.

In other respects both cultures were alike. The smooth culture formed ascospores which could germinate, but conjugation could not be observed. Apparently this strain had already changed from

the haplophase into the diploid phase. Therefore this strain was of no use to me.

The strain of SACCHETTI entered the collection of the yeast-division in 1939, shortly after it had been isolated. In this strain no dissociation had appeared. There was an abundant spore formation. An isogamic or heterogamic conjugation preceded ascus formation.

With this strain ascospores were isolated in maltextract. Nearly all of them germinated. These one-spore cultures were in all respects like each other and like the original strain. Consequently this strain is homozygous, as was to be expected of a *Zygosaccharomyces* species, which has haploid vegetative cells.

Many of the one-spore cultures were transferred to carrot. In some of these cultures, *viz.*, no. 213 and no. 214, conjugation appeared and zygotes were formed. Some of these zygotes were isolated in maltextract. Diploid cultures developed. These cultures again were transferred to carrot. Ascospore formation was abundant, but no conjugation was observed. These cultures fully behaved as a *Saccharomyces* species. They are identical with *Saccharomyces macedoniensis*. Confer the scheme in figure 1.

2. *Saccharomyces macedoniensis.*

Five strains of *S. macedoniensis* were present in the collection of the yeastdivision of the „Centraalbureau voor Schimmelcultures". The strain that was the best in spore formation was chosen [1]).

Ascospores were isolated in maltextract. Only 30 % of the spores germinated. The one-spore cultures were like each other and like the original culture. There were only small differences in growth rate. Consequently the strain of *S. macedoniensis* is also homozygous.

The one-spore cultures were cultivated on carrot. With most cultures nothing specially appeared, but with some cultures a conjugation between the cells was observed and zygotes were formed. In one culture *viz.*, no. 222. besides conjugation also ascospore formation appeared in the zygotes. These ascospores again were isolated 50 % of them germinated. With all the one-spore cultures conjugation preceded ascus formation and ascospores were formed after some days cultivation in maltextract. These cultures fully behaved as a *Zygosaccharomyces* species and were identical with *Zygosaccharomyces Marxianus*. Confer the scheme in figure 2.

[1]) No. 8.58.1 in the yeastcollection of the „Centraalbureau voor Schimmelcultures"

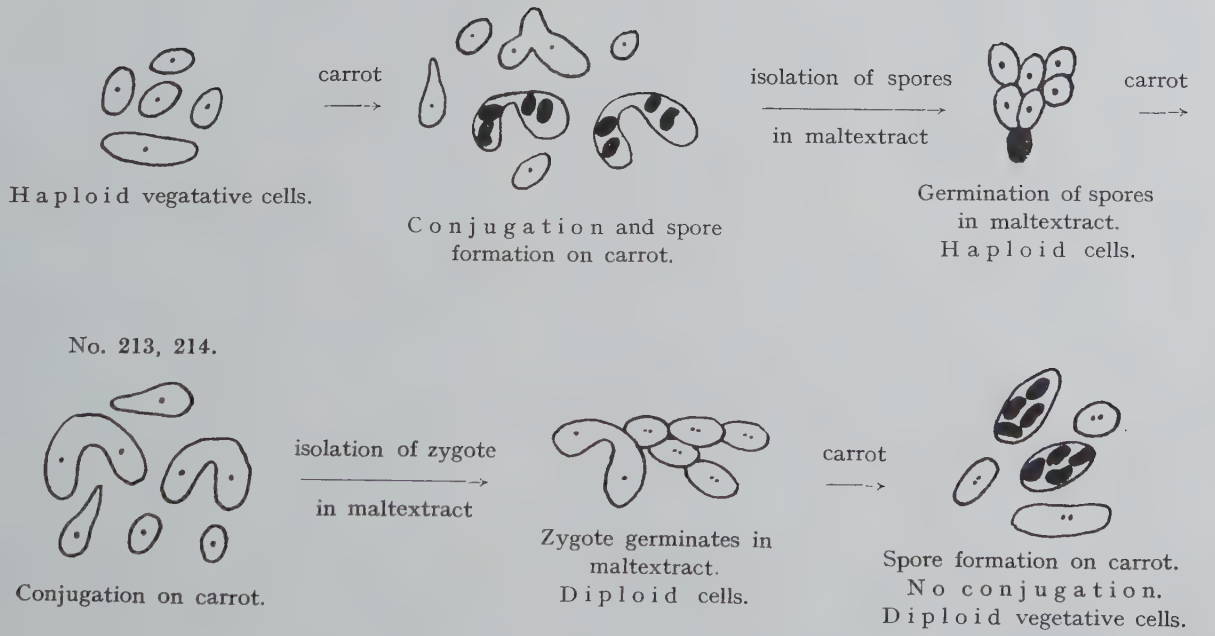

Fig. 1. *Zygosaccharomyces Marxianus.*

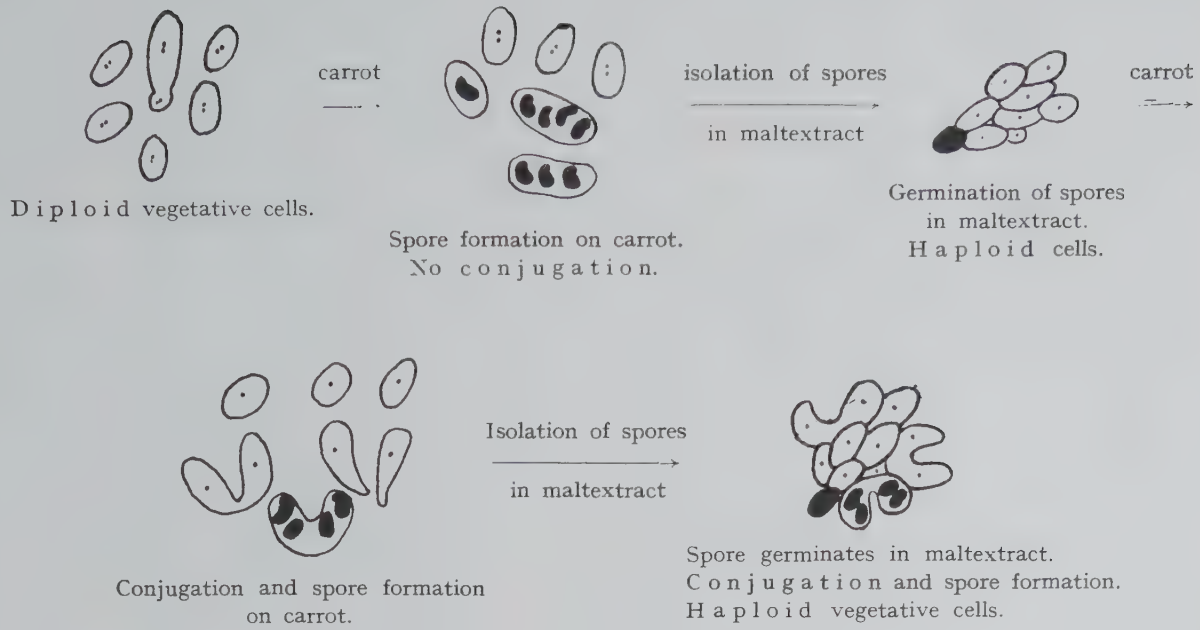

Fig. 2. *Saccharomyces macedoniensis.*

III. Conclusion and Summary.

Zygosaccharomyces Marxianus and *Saccharomyces macedoniensis* belong to the same species. This species is met with in the haplophase (*Z. Marxianus*) as well as in the diplophase (*S. macedoniensis*). It was possible to bring this yeast from the haplophase into the diplophase and vice versa. By keeping this yeast during long times on maltagar it showed a tendency to change from the haplophase into the diplophase, but not into the opposite direction.

It seems quite possible that Hansen, who did not describe a conjugation in this yeast, had met with the diplophase.

It has been once more emphasized — at which Winge and Laustsen and also Lindegren and Lindegren have pointed —, that the genus *Zygosaccharomyces* is no valid genus.

The yeast studied here belongs to the genus *Saccharomyces* and must be designated with the original name given to it by Hansen: *Saccharomyces Marxianus*.

For the sake of completeness it is mentioned here that also an imperfect stage of *S. Marxianus* has been described *viz.*, *Candida macedoniensis* (A. Castellani) Berkhout (1). *Saccharomyces fragrans* Beijerinck has to be considered as its synonym.

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(Biophysical Research Group Utrecht-Delft).

PHOTOSYNTHESIS AS A LIGHT-SENSITIZED TRANSFER OF HYDROGEN

by

E. C. WASSINK

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> "A study of the chemical activities of micro-organisms reveals all the advantages which may be derived from „comparative biochemistry". Although this line of study has not as yet been much developed, it may in future win the same significance for biochemistry as „comparative anatomy" has already long ago attained for anatomy.

The words quoted above were written by A. J. KLUYVER in the preface of his well-known, attractive little book „The chemical activities of Micro-organisms", published in 1931 (1). This book contains a summary of the experiments and considerations presented during the years before by KLUYVER and his collaborators (2, 3, 4), and which, together with observations of other investigators, lead them to consider the transfer of hydrogen as the essential phenomenon in metabolic processes. It may be deemed of special importance in this respect that already in an early stage of the development of these views they were fully aware of the generality of their concept. So, *e.g.*, already in 1926 KLUYVER and DONKER concluded that between dissimilation and assimilation „in physikochemischer Hinsicht durchaus kein Unterschied .... existiert, so dass von vornherein sich nichts dagegen widersetzt, auch die Entstehung der typischen Assimilationsprodukte auf eine Kette katalytischer Wasserstoffübertragungen zurückzuführen". ([4], p. 180). This remarkable statement was based upon the reflexion that in a linked oxido-reduction process, the hydrogen acceptor is energetically raised, also when the total process leads to an overall decrease of free energy. The broad generalisations arising from these concepts went so far that KLUYVER (5, 1) could condense the essence of the overwhelming multitude of biochemical

processes in practically one single general equation, with a few variants; this well-known expression may be represented here once more in its most general basic form:

$$AH + B \rightarrow A + BH \qquad (1)$$

It is clear that these unifying considerations neither could nor would in principle exclude the field of photosynthesis, and it is characteristic that in connection herewith already at an early date KLUYVER's love went especially towards the purple sulphur bacteria. These organisms with their ability to oxidize hydrogen sulphide like colourless sulphur bacteria, but, mysteriously, in the complete absence of oxygen, and their dependence upon the supply of radiant energy like green plants, without, however, developing any oxygen in the light, had since long puzzled the microbiologists, and they seemed well apt to attract the interest of a ,,comparative biochemist''. As a first important step towards a better understanding of this remarkable type of metabolism we may evaluate the remark of KLUYVER and DONKER that from the viewpoint of the hydrogen-transfer concept a dehydrogenation of hydrogen sulphide does not necessarily require oxygen as a hydrogen acceptor, but that another acceptor might come into play as well. The only requirement to be fulfilled is, that the formed acceptor-hydrogen compound can transfer hydrogen to carbon dioxide with the aid of the light ([4], p. 175).

VAN NIEL has taken up these views and condensed them in the following equations

$$\left.\begin{array}{l} 2\,(H_2S + \text{Acceptor} \rightarrow S + \text{Acc.}H_2) \\ 2\,\text{Acc.}\,H_2 + CO_2 \rightarrow 2\,\text{Acc.} + CH_2O + H_2O \end{array}\right\} \qquad (2)$$

$$2\,H_2S + CO_2 \rightarrow 2\,S + CH_2O + H_2O$$

thus expressing the ,,close interrelation of photo- and chemosynthesis'' in these organisms. Starting from KLUYVER's basic equation (*1*) VAN NIEL then derived his general equation for photosynthesis:

$$CO_2 + 2\,AH_2 \rightarrow CH_2O + H_2O + 2\,A \qquad (3)$$

from which both photosynthesis of green plant cells and of purple bacteria could be considered to be special cases. The applicability of the equation (*3*) for the purple sulphur bacteria was then proved by VAN NIEL in quantitative analyses of culture-media (6).

It may be remarked by the way that VAN NIEL already in the discussion of these initial experiments stressed the complete dependence of the CO_2-reduction on the presence of a suitable hydrogen donor (in his cultures *e.g.* H_2S or sulphur), pointing to an interweaving rather than a superposition of — „chemo-" and „photosynthesis". This statement has not always received sufficient attention in the development of the theoretical views lateron (*cf. e.g.* NAKAMURA (7), HANSON (8)).

VAN NIEL's paper can be considered as the photosynthetic „key-stone" to KLUYVER and DONKER's unification of biochemical processes.

It is not the purpose of the present paper to follow in detail the further development of the insight into the photosynthetic mechanism. In the following pages only a few remarks will be presented concerning some points with which the author is somewhat more familiar — or somewhat less unfamiliar — because of experimental work carried on in the last years.

The combined study of photosynthesis and chlorophyll fluorescence in purple sulphur bacteria (9) revealed two independent dark processes in the chain of photosynthesis. From the chief reaction products of the balance equation the hydrogen donor was involved in one of them, carbon dioxide in the other one. The discrimination between these two processes was made possible by the observation of the relations of bacteriochlorophyll fluorescence in the living cells with the course of photosynthesis. It now seems satisfactory that in the past years VAN NIEL from fully different sources of information, chiefly operating with „comparative biochemical" evidence, arrived at the same conclusion. Certain peculiarities of the fatty acid decomposition were independent of the nature of the final hydrogen acceptor, and since light is necessary only in case CO_2 acts as such, it was concluded that the H_2A-decomposition functions without the cooperation of radiant energy ([10], p. 112).

The chief argument VAN NIEL has presented to consider also the CO_2-reduction proper as a dark process is the existence of purple bacteria which can assimilate hydrogen + CO_2 both in the light and in the dark, whereas furthermore the existence of chemoautotrophic bacteria makes it tempting to suppose that the same or at least a closely similar mechanism of carbon dioxide assimilation may be operative in photosynthetic organisms too ([11], p. 347).

The „peculiarities of fatty acid decomposition" VAN NIEL alluded to (10) were especially concerned with the simultaneous use of two of them, each supplied in excess. VAN NIEL found that the velocities of conversion are additive provided the light intensity is sufficiently high. This result differs from the one we found for the simultaneous use of hydrogen and thiosulphate in purple sulphur bacteria ([9], § 11). The most obvious explanation would be that our „high" light intensity was not yet sufficiently high to remove the light as „limiting factor". The high intensity was about 5 times the lower one (about 3.0 and 0.6 $\times$ 10^4 ergs/cm² sec respectively [*l.c.*, p. 314]), whereas the uptake of gas only was between 2 and 3 times. Thus, at the high light intensity the quantum efficiency was about 2/5—3/5 of that at the low intensity, indicating that the system was about half of the time in the BLACKMAN-phase. Following GAFFRON and WOHL (12) this would mean that the rate of gas exchange at our high light intensity still was only half maximal. It was not possible to record in the same experiments the course of photosynthesis for a range of light intensities. But the whole of our experiments made up to that time under these conditions (H_2 or thiosulphate, 29° C., pH 6.3) had given us the conviction that at about 3.0 $\times$ 10^4 ergs/cm² sec (the highest intensity obtainable with our equipment) light saturation was reached fully or at least nearly in most cases. It may well be remarked that GAFFRON and WOHL's statement is not fully general, but presupposes a certain form of the curve of photosynthesis against light intensity with a long transition range between light limitation and light saturation. It doesn't seem excluded that actually this range is much shorter, leading to a half-maximal quantum-efficiency only much nearer to full light saturation. In an „ideal BLACKMAN curve" the quantum efficiency starts to decrease only at light saturation. A full discussion no doubt has to be postponed until further experiments covering a larger variety of conditions will have been performed; those published (9) only formed a first approach in a very limited range of variations. Perhaps, VAN NIEL's observations will form a starting point for a renewed attempt to understand the meaning of the various pigment-protein complexes present in the purple bacteria which question so far had to be left unanswered (*cf.* [9], p. 314, [13]).

The evidence furnished by the simultaneous study of photosynthesis and fluorescence has lead to a scheme ([14], p. 356) in which the „donor-system" and the „CO_2-system" are connected by the „system of energy-transfer" which is thought to be connected with the pigment, and at which light energy enters into the reaction chain. The „problem" of photosynthesis becomes focussed more and more upon what occurs at this last mentioned system. One of the old formulations of this question was whether the pigment participates „chemically" in the reaction or not. From our work (15, 14) we decided to the second alternative, and, as far as the stable primary reaction components H_2A and CO_2 are concerned — the early, crude form in which chemical participation was conceived — I think to day most investigators will agree with this

conclusion. We think that the evidence recapitulated in the beginning of this section strongly supports it. A second question is whether the same thing holds for the pigment-protein complex. I think, this may be answered in the affirmative: the pigment-protein complex may no doubt be discriminated from the very probably also proteinaceous dark systems at which in purple sulphur bacteria H_2A and CO_2 react. For one substance, likely to act as a hydrogen donor, namely water, it has, also in recent times be admitted that it indeed reacts at the pigment system. In important schemes of photosynthesis, *e.g.* the one of VAN NIEL (16) it even plays a central rôle, since the photic decomposition of some sort of pigmenthydrate was conceived as a common feature of various types of photosynthesis. We have adopted a related view for green plant photosynthesis, however, with a shift of stress from the pigment to the protein of the pigment-protein-complex, symbolizing the energy acceptor by RHOH (15, 17). For purple bacteria the introduction of HOH at this place already appears much less obligatory (*cf.* also [9], p. 350) but it may well be emphasized that, also for green cells it is not at all proved. It is *e.g.* equally well conceivable that also in this case the hydrogen donor underlies a primary conversion in a dark process. It will, however, not be easy to demonstrate the place of the water as hydrogen donor in the photosynthetic process since it cannot be supplied or removed at will. Already a number of years ago TRELEASE and collaborators (18, 19) made interesting observations which may perhaps be apt to initiate a further development of this problem. They observed that replacement of ordinary by heavy water resulted in a „BLACKMAN"-inhibitation of *Chlorella*-photosynthesis, and they considered this result to be in favour of the assumption that water reacts in a dark process. This interpretation seems correct in a general form, but the result is not equally unambiguous as those obtained with purple bacteria in which the supply of hydrogen donor is controlled. For, except in a possible specific dark conversion reaction, water no doubt takes part in a more indirect way in various other parts of the reaction chain. One would be interested to know what is the behaviour of fluorescence upon replacement of ordinary by heavy water. If it were of the type observed in purple bacteria upon dosage of the hydrogen donor (9), the chances upon a specific meaning of TRELEASE's results would appear increased.

In our first work on chlorophyll-fluorescence of *Chlorella* (20) we in general observed no definite reactions of fluorescence upon the reach of light satu-

ration in photosynthesis. JOHNSTON and MYERS (21), however, have expressed the opinion that our experiments may have been confined to a too limited range of conditions. This may in part be the case, since at that time our equipment for fluorescence measurements was still in an early stage of development. An extension of these observations to a larger variety of conditions, especially to still higher light intensities, and including the influence of CO_2 has since long been planned, but the limited scope of our group so far prevented to carry it out, other questions appearing of still more importance. Some incidental observations were made (WASSINK and KERSTEN, unpublished) showing that at high light intensities a decrease of temperature may cause an increase of fluorescence. However, only in a combined systematic study of fluorescence and gas exchange such observations may be evaluated for the elucidation of parts of the mechanism as was particularly shown in a study with diatoms (22).

Considering the facts, no direct evidence seems available that H_2O is dehydrogenated directly with the aid of light energy at the energy-transfer system. Nevertheless, the function of the energy absorbing system can hardly be anything else than to enable and promote the transfer of hydrogen from the „donor-system" to the „CO_2-system". The sequence of reactions may in a simple and general form perhaps be symbolized as follows:

„Donor-System"	Pigment System (= System of Energy transfer)	„CO_2-System"
$R'+DH \rightarrow R'DH$	$R''P+EH \rightarrow R''PEH$	$R'''G+CO_2 \rightarrow R'''GCO_2$
$R'DH+E \rightarrow EH+R'D$	$R''P+h\nu \rightarrow R''P^*$	$R'''GCO_2+[4\,FH] \rightarrow$
$R'D \rightarrow R'+D$	$R''P^* \rightarrow R''P+h\nu'$ (fluorescence)	$R'''GCH_2O+H_2O+4\,F$
	$R''P^* \rightarrow R''P+\epsilon$ (heat)	
	$R''P^*EH+F \rightarrow R''PE+FH$ (energy transfer)	$R'''GCH_2O \rightarrow R'''G+CH_2O$
	$R''PE \rightarrow R''P+E$	

R', R'', R''' are proteins of catalysts.
D, E, F and G reactive groups or carrier molecules.
P pigment. P* = activated pigment molecule.

Abstraction has been made of the eventual occurrence of polymerization reactions at the CO_2-system, and of the possibility that at certain points O_2 may enter as a concurring hydrogen acceptor. [4 FH]: very probably entering stepwise into the reaction.

The nature of the „carrier" substances in the various links is left aside since they are still unknown. It seems well conceivable that *e.g.* a phosphate-cyclus may be interwoven in the course of reactions, as some investigators are willing to assume in view of the important discoveries made in this respect by VOGLER and UMBREIT in connection with chemosynthesis (*cf.* [11]).

In view of a possible rôle of phosphate-compounds in photosynthesis, we may draw attention to LIPMANN's formulation (23) of exchange-reactions between „water-" and „phosphate"-systems, of the following type

$$R.COOH + H_2X \rightarrow RCH(OH)_2 + X$$
$$RCH(OH)_2 + HO\text{-}ph \rightarrow RCH(OH)\,(O\text{-}ph) + H_2O.$$

The „energy-acceptor" R"P EH might have the nature of R"PCH(OH) (O–ph), and the energy transfer reaction might then be of the type:

$$R''P^*.CH(OH)\,(O\text{-}ph) + F \rightarrow R''P\overset{\overset{O}{//}}{C}\text{—}O\text{-}ph + FH_2.$$

But these considerations are so far purely hypothetical, and we will not dwell further upon them and their consequences.

In any case it doesn't seem obligatory to consider the substance „EH" as „water"; like already remarked, the decomposition of water in green plant cells may as well take place already at a „donor-system", operating in the dark. Neither is it necessary to postulate that the substance EH is exactly the same in all types of photosynthetic organisms. This even doesn't appear very probable, *e.g.*, for green plant cells and purple bacteria, in view of the different properties of the pigment-protein system. It seems not unlikely that EH is analogous but not alike in these cases.

The mode of cooperation of the pigment in the reaction may at present seem most likely to be a „sensitisation" or energy transfer as far as the pigment molecules as such are concerned, but at the pigment-protein complex an oxidation reduction reaction in which only intracellular carrier substances react, may take place under uptake of the transferred light energy. The pigment-protein complex may well be denoted as a photodehydrogenase or, including the energy acceptor, as a photo-oxydo-reduction system, forming an intermediate between the two poles of the hydrogen transfer chain from the hydrogen donor to carbon dioxide.

The existence of this hydrogen transfer chain and the various ways in which it is influenced by the „pushing" of the hydrogen donor, and the „pulling" of CO_2 are demonstrated in an especially impressive manner by the study of redox potentials in relation to photosynthesis. More than 10 years ago ROELOFSEN (24), working in KLUYVER's laboratory, carried out some preliminary experiments with suspensions of *Chromatium*, strain D. A more thorough investigation was since long planned in our group (*cf.* [14]) and is now in progress. We hope to be able to present lateron a detailed report: for our present purpose we will discuss only a few observations.

Simultaneous measurements of photosynthesis and the course of the redox potentials were carried out with suspensions of *Chromatium*, strain D. Ordinary WARBURG manometers were used, provided with cylindrical vessels of about 50 ccm contents. The ground joint was replaced by a tightly fitting rubber stopper which has several holes to take up the manometer capillary, a liquid joint tube (KCl-agar), a removable glass-stopper (for ventilation of the vessel) and 3 glass tubes with sealed platinum electrodes. The electrodes consist of platinum wires, about 1 cm long and 0.2 mm thick. They are prepared with great care, thoroughly cleaned before use; the preparatory heating of the tube and the sealing is wholly done with an alcohol-flame. After each experiment the electrodes are thoroughly cleaned. As a rule 3 vessels (containing 3 electrodes each) are used parallelly, allowing three different conditions. The 3 electrodes in each vessel permit to recognize unsuitable ones, which can easily be substituted. Twenty ccm of suspension are introduced into each vessel. Potentials are determined at each electrode separately with the aid of a 10-pole commutator, against a saturated calomel electrode, using a COLEMAN pH-meter 3 D as measuring device. The incident light intensity is about 3×10^4 ergs/cm^2 sec. sodium light.

We will confine ourselves here to some observations made with hydrogen. This was chosen as a hydrogen donor because it doesn't leave oxidation products in the solution which may exert unknown influences upon the electrodes. Experiments in phosphate buffer at pH 6.6 clearly show an increase of potential (shift towards the oxidized side) upon illumination. But, very characteristic differences occur according to the conditions under which the bacteria are illuminated. The strongest shift occurs when only $N_2 + 5\ \%\ CO_2$ is supplied, with $N_2 + 5\ \%\ CO_2 + 30\ \%\ H_2$ the shift is much smaller, whereas with $N_2 + 15\ \%\ H_2$ it is still smaller. A characteristic example is given in Fig. 1.

It is clearly shown that there is no direct correlation between the velocity of photosynthesis and the potential change. This indicates that the potential change is not caused by the formation of certain final products or waste products of photosynthesis, and it may be assumed that it actually represents the state of oxido-reduction of essential redox-systems of the photosynthetic apparatus. Moreover this is shown by the fact that the potential change is reversed when the light is turned off. Additionally Fig. 1 shows that during the illumination the potential is fairly constant in the vessel which had received $H_2 + CO_2$, whereas it tends to increase slowly in the vessel with CO_2, and to decrease in the vessel with H_2. This is exactly what may be expected when the potentials reflect the state of oxido-reduction of the hydrogen transferring systems. In the dark a certain state of reduction is reached, that probably can be

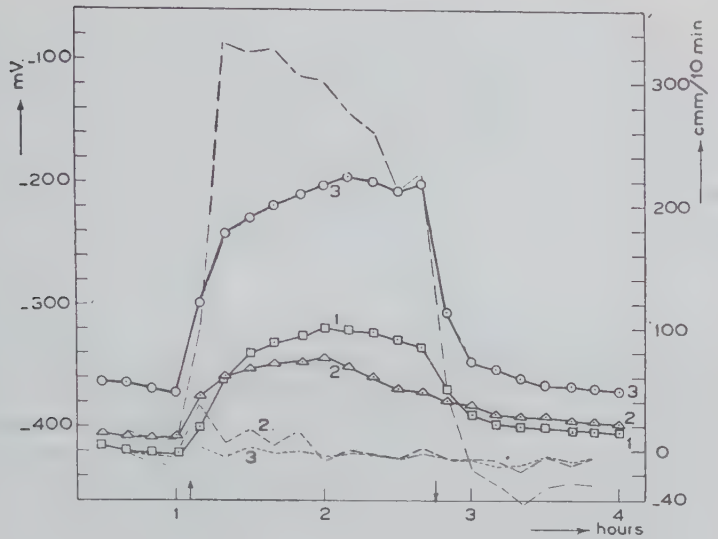

Fig. 1.

Abscissa : Time in hours.
Ordinates : Potential difference in millivolts (mV) against saturated KCl-calomel electrode (left scale).
Uptake of gas (CO_2, H_2, or $CO_2 + H_2$) in cmm/10 min. (right scale).
Potentials : Full drawn curves with marks.
Gas exchange : interrupted curves.
↑: light on, ↓: light off.
Suspension medium: phosphate buffer 1/15 mol + 0.6 % NaCl, pH 6.6.
1: N_2 – 30 % H_2 – 5 % CO_2, 1 vessel, average of 3 electrodes.
2: N_2 – 15 % H_2 , 1 ,, ,, ,, 3 ,,
3: N_2 – 5 % CO_2 , 1 ,, ,, ,, 3 ,,
Per vessel 20 ccm of medium, containing 104 cmm of cells.
Exp. of 12.12.'45.

practically fully maintained on intracellular hydrogen donors since it doesn't differ much with and without hydrogen in the gas phase. Upon illumination the mechanism of hydrogen transfer from the donors to CO_2 comes into action. At the CO_2-catalyst the hydrogen is irreversibly bound in the final products of photosynthesis. If sufficient CO_2 is present the reduced side of the various links of the hydrogen transfer chain will quickly be dehydrogenated, and if no sufficient amounts of hydrogen donors are available, in the light the various systems will shift strongly towards the oxidized side. In as much as the available intracellular supply of hydrogen becomes exhausted this shift will tend to go farther during illumin-

ation, which reflects in a gradual further increase of the potential.

If a hydrogen donor is supplied in excess, but no CO_2 is given, the reverse situation holds. After the start of an illumination the potential rises much less than in the above discussed case: much hydrogen can be supplied, little removed owing to the lack of acceptors. It is very characteristic that a potential decrease sets in in the light as soon as the acceptors become completely exhausted. Practically the same level is reached as after darkening. It appears that under these conditions the supply of hydrogen by the dehydrogenases can master the removal going on in the light. When CO_2 and H_2 are supplied both, a situation intermediate between the two ones discussed develops upon illumination. At first the rise of potential is much like that with H_2 alone, but it goes to a higher level and doesn't tend to decrease owing to the fact that the hydrogen acceptors also are constantly available anew, because ultimately the hydrogen is irreversibly transferred to CO_2.

So far the behaviour of the redox potential appears rather intelligible, but if wider variations of conditions are studied, the phenomena are apparently complicated by changes in the state of activation of the various catalysts. In a previous study (9) it was already observed that especially the pH of the suspension medium strongly influences the reactions of the ,,donor-system''. So, *e.g.*, with pH increasing from 6.2—7.6, the reactivity towards hydrogen was found increased (*cf. e.g.* [9], fig. 26).

A study of the influence of pH on the redox potential is now in progress; the results obtained so far indicate that with increasing pH-values the reducing tendencies of the cells become more pronounced, in accordance with the conclusion drawn from the above mentioned earlier experiments. Here we will not yet discuss this material in detail, but draw attention to a fact of special interest, *viz.*, that in the absence of CO_2 but in the presence of a fairly high hydrogen tension in various cases the potential d e c r e a s e s upon illumination. The most obvious examples of this behaviour were so far found in non-buffered media, at pH $\sim$ 8.0 (sterilized solutions of NaCl, 2 %, showing about this pH), but also in phosphate buffer-solutions of pH $\sim$ 7.5 potential decreases were found upon illumination in the presence of H_2, when CO_2 was absent. Fig. 2 shows a.o. some of such curves obtained in non-buffered medium. With CO_2 alone (bicarbonate was added to the suspension medium) a potential rise occurs of the same type as shown in Fig. 1. With $CO_2 + H_2$ the potential change is only very small, notwith-

standing the active gas exchange. This shows that hydrogen can be supplied at a rate sufficient to maintain about the same state of reduction as in the dark. In pure N_2 – NaCl the course of the potential is much the same as in NaCl – $NaHCO_3$ – N_2 - 5 % CO_2.

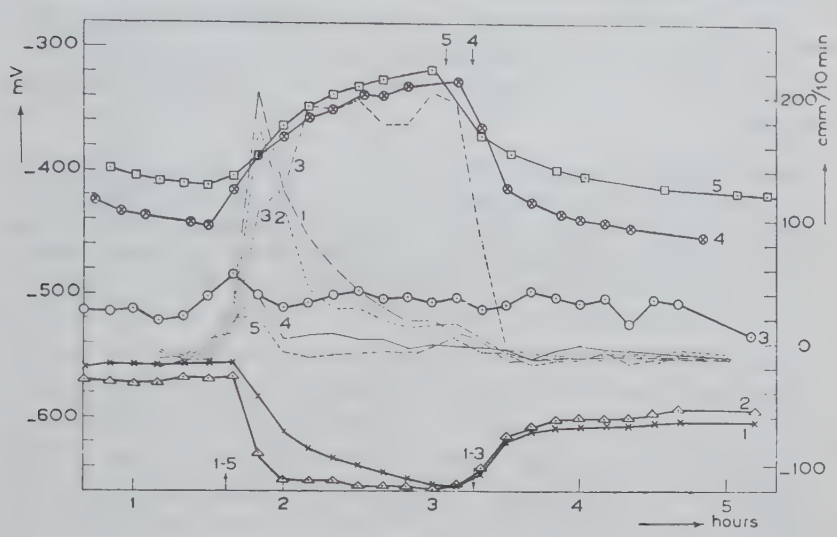

Fig. 2.

General legenda *cf.* Fig. 1.

1: N_2 – 15 % H_2, suspension medium 2 % NaCl, 1 vessel, average of 3 electrodes.

2: N_2 – 30 % H_2, suspension medium 2 % NaCl, 1 vessel, average of 3 electrodes.

3: N_2 – 30 % H_2. 5 % CO_2, suspension medium 2 % NaCl 0.7 % $NaHCO_3$; 1 vessel, average of 3 electrodes. Nos 1—3: Exp. of 4.3.'46.

4: N_2 – 5 % CO_2, suspension medium 2 % NaCl + 0.7% $NaHCO_3$, average of 3 vessels, 9 electrodes; Exp. of 1.3.'46.

5: N_2, suspension medium 2 % NaCl, average of 3 vessels, 9 electrodes; Exp. of 28.2.'46.

Nos 1—5: Per vessel 20 ccm of medium, containing 73 cmm of cells; initial pH ∽ 8.0.

The conditions to which the potential decrease upon illumination in the presence of H_2 is bound have not yet all been elucidated. It might be asked whether a change in pH can be a factor influencing the observed potentials. This seems not likely but nevertheless it is felt necessary to follow the pH during the experiment. So far, however, we had not yet the opportunity to carry this through. Provisionally we should like to ascribe the observed potential decrease to an active promotion by the light of the reducing capacities in the chain of photosynthesis. This is not entirely new.

GAFFRON (*cf. e.g.* [25]) with his adapted algae found an activation of hydrogen evolution upon illumination under N_2, and some years earlier we found an indication for a direct reducing action of the light in the rise of fluorescence during illumination in suspensions of *Chlorella* in which the evolution of O_2 was poisoned by KCN (17). The observed course of the redox potential appears to be a first demonstration of an analogous effect of the light in purple sulphur bacteria.

It is tempting to discuss still other of the widely varied aspects the problem of photosynthesis offers to day. It would be especially tempting to dwell upon the comparative physiology of the pigment systems in various classes of photosynthetic organisms, which, from a view point of energy transfer offer very interesting features (*cf. e.g.* [26]). But the scope available for this paper is limited, and thus, other points will have to be treated elsewhere.

The considerations presented may have emphasized the useful service that KLUYVER's concept of hydrogen transfer has rendered to the study of photosynthesis, and that from the further course of this study it mutually receives constantly new support. The new observations discussed in this paper may show also for photosynthesis the close connection between essential features of the complex of reactions going on in a special case, and the redox potential. The results obtained so far still are of a somewhat preliminary nature but they may well serve to indicate that also for this field another of the ideas that emerged largely from KLUYVER's school may become fruitful in the future, *viz.*, the characterization of a special type of metabolism by a special redox potential: „dass ein Zusammenhang besteht zwischen den in Medien stoffwechselnder Zellen auftretenden Oxydoreduktionspotentialen und der Natur des Stoffwechsels der betreffenden Zellen" ([27], p. 1).

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